

GEMINIVIRUS RESISTANT TRANSGENIC PLANTS

Related Applications

This is a continuation-in-part application of U.S. application serial number 09/289,346, filed April 9, 1999, pending, which claims priority to U.S. provisional application serial number 60/125,004, filed March 18, 1999, the entire contents of each of which are incorporated by reference herein.

Statement of Government Support

Research directed to this invention is supported in part by USDA Grant No. NRI-2001-02619. The Government has certain rights in this invention.

Field of the Invention

The present invention relates to transgenic plants with increased resistance to geminivirus infection, and mutants of the AL1/C1 (Rep) geminivirus protein useful for producing such plants. Methods of screening for suitable mutants are also provided.

Background of the Invention

The geminiviruses are a large and diverse family of plant DNA viruses, with circular single-stranded (ss) DNA genomes that replicate through circular double stranded DNA intermediates. See Hanley-Bowdoin et al., *Cri. Rev. Plant Sci.* 18:71 (1999); Lazarowitz, *Crit. Rev. Plant Sci.* 11:327 (1992); Timmermans et al., *Annu. Rev. Plant Physiol.* 45:79 (1994). Viral DNA replication, which results in both single and double stranded viral DNAs in large amounts, involves the expression of only a small number of viral proteins that are involved in either replication or viral transcription. The geminiviruses appear to rely primarily on the machinery of the host to copy their genomes and express their genes, including the nuclear DNA and RNA polymerases of their plant hosts. These properties of geminiviruses are unusual among plant viruses, most of which are RNA viruses or replicate through RNA intermediates using virus-encoded replicases. Geminiviruses infect a broad variety of plants and cause significant crop losses worldwide.

Geminiviruses are subdivided on the basis of host range in either monocots or dicots, genome structure, and insect vector. Subgroup I geminiviruses (also known as Mastreviruses) are transmitted by leafhoppers and infect primarily monocots, although Subgroup I geminiviruses that infect dicots are known. Subgroup II geminiviruses (also known as Curtoviruses) are transmitted by leafhoppers and infect dicots. Subgroup III geminiviruses (also known as Begomoviruses) are transmitted by whiteflies and infect dicots. Subgroup I & II viruses have genomes comprising a single ssDNA component; Subgroup III geminiviruses typically have a bipartite genome comprising two similarly sized DNAs (usually termed A and B), as illustrated by African cassava mosaic virus (ACMV), tomato golden mosaic virus (TGMV) and potato yellow mosaic virus. However, monopartite geminiviruses that infect dicots are known, for example Tomato Yellow Leaf Curl Virus (TYLCV). The genomes of monopartite Subgroup II and III geminiviruses have an arrangement of genes similar to the AL1, AL2 and AL3 genes found on the A DNA component of bipartite Subgroup III geminiviruses.

Subgroup III viruses are also divided into "old world" and "new world" viruses, a division based on evolutionary divergence.

For successful infection of plants by bipartite geminiviruses, both the A and B genomic components are required. Sequence analysis of the two genome components reveals six open reading frames (ORFs). Four of the ORFs are encoded by DNA A and two by DNA B. On both components, the ORFs diverge from a conserved 230 nucleotide intergenic region (common region) and are transcribed bidirectionally from double stranded replicative form DNA. The ORFs are named according to genome component and orientation relative to the common region (i.e., left versus right (L/R), or virion versus complementary sense (V/C)). Certain proteins are known to be involved in the replication of viral DNA (REP genes). *See, e.g., Elmer et al., Nucleic Acids Res. 16:7043 (1988); Hatta and Francki, Virology 92:428 (1979).*

The A genome component contains all viral information necessary for the replication and encapsidation of viral DNA, while the B component encodes functions required for movement of the virus through the infected plant. The DNA A component of these viruses is capable of autonomous replication in plant cells in the absence of DNA B when inserted as a greater than full length copy into the genome of plant cells, or when a copy is transiently introduced into plant cells. In monopartite geminivirus genomes, the single genomic

component contains all viral information necessary for replication, encapsidation, and movement of the virus.

Geminiviruses cause substantial losses among economically important crops, including tomato, bean and cucurbit. Current strategies to control geminivirus infections target the insect vectors that carry the viruses. However, the use of insecticides to control or combat a geminivirus infection can be expensive and inefficient. Additionally, insect hosts can vary in their susceptibility to available insecticides, and resistance to insecticides can develop over time. *See Markham et al., Pestic. Sci. 42:123 (1994).*

Varied approaches have been used in attempts to generate geminivirus-resistant plants, including classical breeding and transgenic approaches, with limited success. Unlike plant RNA viruses, the introduction of geminivirus sequences into transgenic plants does not confer resistance, and conversely, frequently results in the production of functional viral proteins (Hayes and Buck, *Nucleic Acids Res. 17:10213 (1989)*; Hanley-Bowdoin et al., *Proc. Natl. Acad. Sci. USA 87:1446 (1990)*). Kunik et al. describes transgenic tomatoes that contain a geminivirus coat protein gene (Kunik et al., *BioTechnology 12:500 (1994)*). Expression of antisense RNAs against geminivirus replication proteins in transgenic plants reduces the level of viral DNA accumulation up to 70% (Day et al., *Proc. Natl. Acad. Sci. USA 88:6721 (1991)*), to a level that is still sufficient to confer wild type viral symptoms (Hanley-Bowdoin et al., *Plant Cell 1:1057 (1989)*). Similarly, the presence of defective-interfering replicons in transformed plants can reduce the level of viral DNA accumulation by about 70% (Frischmuth and Stanley, *Virology 200:826 (1994)*). The antisense RNAs and defective-interfering replicons function best against their cognate viruses (Bejarano et al., *Plant Mol. Biol. 24:241 (1994)*), further limiting their usefulness. Antisense RNA targeted to mRNA of the Rep protein (encoded by the C1 gene) was used to produce transgenic *Nicotiana benthamiana* plants with altered responses to TYLCV. (Bendahmane and Gronenborn, *Plant Mol. Biol. 33:351 (1997)*).

Accordingly, it is desirable to devise new strategies to control geminivirus infection.

Summary of the Invention

A first aspect of the present invention is a plant comprising transformed plant cells, said transformed plant cells containing a heterologous nucleic acid construct comprising, in the 5' to 3' direction, a promoter operable in said plant cells, a nucleic acid sequence encoding a mutant AL1 protein, where said nucleic acid sequence is located downstream from said

promoter and operatively associated therewith, and comprising a mutation in the Rb binding region, whereby binding of said mutant AL1 protein to a plant Rb protein is reduced compared to binding which would occur in the presence of a wild-type AL1 protein; and a mutation in the AL1 protein, whereby said mutant AL1 protein suppresses viral replication compared to that which would occur in the presence of a wild-type AL1 protein; and a termination sequence positioned downstream from said nucleic acid sequence and operatively associated therewith, wherein expression of said mutant AL1 protein increases resistance of said plant to infection by at least one geminivirus, compared to a non-transformed control.

A further aspect of the present invention is a method of making a transgenic plant having increased resistance to geminivirus infection. The method comprises providing a plant cell capable of regeneration; transforming the plant cell with a DNA construct comprising, in the 5' to 3' direction, (a) a promoter operable in said plant cell, (b) a nucleic acid sequence encoding a mutant AL1 protein, said nucleic acid sequence located downstream from said promoter and operatively associated therewith, and comprising i) a mutation in the Rb binding region, whereby binding of said mutant AL1 protein to a plant Rb protein is reduced compared to binding which would occur in the presence of a wild-type AL1 protein; and ii) a mutation in the AL1 protein, whereby said mutant AL1 protein suppresses viral replication compared to that which would occur in the presence of a wild-type AL1 protein; and (c) a termination sequence positioned downstream from said nucleic acid sequence and operatively associated therewith; and then regenerating a transgenic geminivirus-resistant plant from said transformed plant cell, wherein expression of said mutant AL1 protein increases resistance of said plant to infection by at least one geminivirus, compared to a non-transformed control.

A further aspect of the present invention is a nucleic acid construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a promoter operable in a plant cell, a nucleic acid sequence encoding a mutant AL1 protein, said nucleic acid sequence located downstream from said promoter and operatively associated therewith, and comprising a mutation in the Rb binding region, whereby binding of said mutant AL1 protein to a plant Rb protein is reduced compared to binding which would occur in the presence of a wild-type AL1 protein; and ii) a mutation in the AL1 protein, whereby said mutant AL1 protein suppresses viral replication compared to that which would occur in the presence of a wild-type AL1 protein; and a termination sequence positioned downstream from said nucleic acid sequence and operatively associated therewith.

A further aspect of the present invention is a method of producing nucleic acid constructs useful in conferring increased geminivirus-resistance to plants, comprising, screening mutants of a geminivirus AL1 protein to identify mutations that suppress the ability of the AL1 protein to bind to a plant Rb protein; preparing a nucleic acid molecule encoding an AL1 protein having said mutation, and further having a mutation that suppresses geminivirus replication compared to that which would occur in the presence of a wild-type AL1 protein; and preparing a nucleic acid construct comprising, in the 5' to 3' direction, a promoter operable in a plant cell, a nucleic acid sequence encoding said mutant AL1 protein, said nucleic acid sequence located downstream from said promoter and operatively associated therewith, and a termination sequence positioned downstream from said nucleic acid sequence and operatively associated therewith.

A further aspect of the present invention is a nucleic acid construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction: a promoter operable in a plant cell, a nucleic acid sequence encoding a mutant AL1 protein, said nucleic acid sequence located downstream from said promoter and operatively associated therewith, and comprising: a mutation in the oligomerization domain, whereby binding of said mutant AL1 protein to wild type AL1 protein is reduced compared to binding which would occur with a wild-type AL1 protein; and a mutation in the AL1 protein, whereby said mutant AL1 protein suppresses viral replication compared to that which would occur in the presence of a wild-type AL1 protein; and a termination sequence positioned downstream from said nucleic acid sequence and operatively associated therewith.

The present invention further provides a plant comprising transformed plant cells, said transformed plant cells comprising a heterologous nucleic acid construct encoding a mutant AL1 protein, wherein said heterologous nucleic acid construct comprises a nucleotide sequence such as SEQ ID NO:40 (K144), SEQ ID NO:41 (E145), SEQ ID NO:42 (E146), SEQ ID NO:43 (EE146), SEQ ID NO:44 (A147Y), SEQ ID NO:45 (L148), SEQ ID NO:46 (L148V), SEQ ID NO:47 (L148V*), SEQ ID NO:48 (L148G), SEQ ID NO:106 (L145A), and SEQ ID NO:49 (II151) or any combination thereof. The plant of this invention can further comprise a nucleotide sequence such as SEQ ID NO:28 (Ala1), SEQ ID NO:32 (Ala5), SEQ ID NO:33 (Ala6), SEQ ID NO:34 (Ala7), SEQ ID NO:36 (Ala9), SEQ ID NO:26 (Ala13), SEQ ID NO:38 (Leu), SEQ ID NO:29 (Ala2), SEQ ID NO:30 (Ala3), SEQ ID NO:31 (Ala4), SEQ ID NO:39 (Ala4+5), SEQ ID NO:27 (Ala14), SEQ ID NO:35 (Ala8), and SEQ ID NO:37 (Ala10) or any combination thereof.

In addition, the present invention provides a method of making the transgenic plant of this invention, comprising: a) transforming a cell of a plant with a heterologous nucleic acid construct encoding a mutant AL1 protein, wherein said heterologous nucleic acid construct comprises a nucleotide sequence such as SEQ ID NO:40 (K144), SEQ ID NO:41 (E145), SEQ ID NO:42 (E146), SEQ ID NO:43 (EE146), SEQ ID NO:44 (A147Y), SEQ ID NO:45 (L148), SEQ ID NO:46 (L148V), SEQ ID NO:47 (L148V*), SEQ ID NO:48 (L148G) SEQ ID NO:106 (L145A), and SEQ ID NO:49 (II151) or any combination thereof; and b) regenerating the transgenic plant from said transformed plant cell. The heterologous nucleic acid construct of this method can further comprise a nucleotide sequence such as SEQ ID NO:28 (Ala1), SEQ ID NO:32 (Ala5), SEQ ID NO:33 (Ala6), SEQ ID NO:34 (Ala7), SEQ ID NO:36 (Ala9), SEQ ID NO:26 (Ala13), SEQ ID NO:38 (Leu), SEQ ID NO:29 (Ala2), SEQ ID NO:30 (Ala3), SEQ ID NO:31 (Ala4), SEQ ID NO:39 (Ala4+5), SEQ ID NO:27 (Ala14), SEQ ID NO:35 (Ala8), and SEQ ID NO:37 (Ala10) or any combination thereof.

Also provided herein is an isolated nucleic acid comprising a nucleotide sequence encoding a mutant AL1 protein, wherein the nucleotide sequence can be SEQ ID NO:40 (K144), SEQ ID NO:41 (E145), SEQ ID NO:42 (E146), SEQ ID NO:43 (EE146), SEQ ID NO:44 (A147Y), SEQ ID NO:45 (L148), SEQ ID NO:46 (L148V), SEQ ID NO:47 (L148V*), SEQ ID NO:48 (L148G) SEQ ID NO:106 (L145A), and SEQ ID NO:49 (II151) or any combination thereof. The nucleic acid construct can also comprise a nucleotide sequence such as SEQ ID NO:28 (Ala1), SEQ ID NO:32 (Ala5), SEQ ID NO:33 (Ala6), SEQ ID NO:34 (Ala7), SEQ ID NO:36 (Ala9), SEQ ID NO:26 (Ala13), SEQ ID NO:38 (Leu), SEQ ID NO:29 (Ala2), SEQ ID NO:30 (Ala3), SEQ ID NO:31 (Ala4), SEQ ID NO:39 (Ala4+5), SEQ ID NO:27 (Ala14), SEQ ID NO:35 (Ala8), and SEQ ID NO:37 (Ala10) or any combination thereof.

Furthermore, the present invention provides a mutant AL1 protein comprising an amino acid sequence selected from the group consisting of SEQ ID NO:17 (K144), SEQ ID NO:18 (E145), SEQ ID NO:19 (E146), SEQ ID NO:20 (EE146), SEQ ID NO:21 (A147Y), SEQ ID NO:22 (L148), SEQ ID NO:23 (L148V), SEQ ID NO:24 (L148G), SEQ ID NO:25 (II151), SEQ ID NO:109 (L145A), SEQ ID NO:3 (Ala1), SEQ ID NO:15 (Ala5), SEQ ID NO:5 (Ala6), SEQ ID NO:6 (Ala7), SEQ ID NO:8 (Ala9), SEQ ID NO:9 (Ala13), SEQ ID NO:11 (Leu), SEQ ID NO:12 (Ala2), SEQ ID NO:13 (Ala3), SEQ ID NO:14 (Ala4), SEQ ID NO:4 (Ala4+5), SEQ ID NO:10 (Ala14), SEQ ID NO:7 (Ala8), and SEQ ID NO:16 (Ala10), as well as an isolated nucleic acid encoding any of the proteins of this invention.

Brief Description of the Drawings

Figure 1 shows the amino acid sequence of TGMV Rep, from amino acid 110-179, and the sequences of alanine and leucine substitution mutants. Underlined amino acids correspond to a conserved helix-loop-helix motif found in the Rep proteins of all geminiviruses; double underlined amino acids show the substitutions.

Figure 2A diagrams the AL1 protein, showing the positions of the three conserved cleavage motifs (solid boxes), two predicted pairs of helices (hatched circles), and the ATP binding site (hatched box). The domains for DNA binding and cleavage/ligation activity are indicated by solid lines and the oligomerization domain is shown as a dashed line. Solid lines below the AL1 diagram mark the sizes of the truncated proteins and are designated by their N- and C-terminal amino acids. The boxed region indicates the limits of the core oligomerization domain.

Figure 2B shows the results of an immunoblot assay to detect protein interactions of C-terminal truncated proteins with full length GST-AL1. Total extracts from insect cells co-expressing GST-AL1 with truncated AL1 proteins were incubated with glutathione-sepharose, washed and eluted in SDS-sample buffer. Bound AL1 proteins were visualized by immunoblot assays. Input (lanes 1-3) and bound (lanes 4-6) fractions were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting. AL1 1-180 (lanes 1 and 4), AL1 1-168 (lanes 2 and 5), AL1 1-158 (lanes 3 and 6).

Figure 2C shows the results of an immunoblot assay to detect protein interactions of N-terminal truncated proteins with full length GST-AL1. Total extracts from insect cells co-expressing GST-AL1 with truncated AL1 proteins were incubated with glutathione-sepharose, washed and eluted in SDS-sample buffer. Bound AL1 proteins were visualized by immunoblot assays. Input (lanes 1-3) and bound (lanes 4-6) fractions were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting. AL1 134-352 (lanes 1 and 4), AL1 147-352 (lanes 2 and 5) and AL1 159-352 (lanes 3 and 6).

Figure 2D shows the results of co-purification assays performed with GST-AL1 119-180 and N-terminal truncated AL1 119-352 (lanes 1 and 5), AL1 134-352 (lanes 2 and 6), AL1 147-352 (lanes 3 and 7), and AL1 158-352 (lanes 4 and 8).

Figure 3 shows mutations generated in the oligomerization domain of AL1, and their characteristics.

Figure 4A shows oligomerization properties of AL1 proteins with mutations in the

core oligomerization domain in insect cells. Protein interactions were assayed as described in **Figure 1**. Mutant AL1 proteins co-expressed with full length GST-AL1 were extracted (top) and bound to glutathione-sepharose (bottom). Lanes correspond to wild type AL1 (lane 1), AL1 E--N140 (Ala4, lane 3), AL1 KEE146 (Ala5, lane 4), AL1 REK154 (Ala6, lane 5), AL1
 5 EKY159 (Ala7, lane 6), AL1 Q-HN165 (Ala8, lane 7), AL1 N-DR172 (Ala9, lane 8), and AL1 K-E179 (Ala10, lane 9). Wild type AL1 was also co-expressed with GST alone (lane 2).

Figure 4B shows oligomerization properties of AL1 proteins with mutations in the core oligomerization domain in insect cells. Protein interactions were assayed as described in **Figure 1**. Mutant AL1 proteins were co-expressed with GST-AL1 119-180. The lanes are as
 10 described in panel A.

Figure 4C shows the mutant AL1 EKY159 co-expressed with GST-AL1 fusion proteins of full-length and truncated AL1. Lanes 1-4 show AL1 proteins bound to glutathione-sepharose. The GST-AL1 proteins assayed were full-length GST-AL1 (lanes 1 and 5), GST-AL1(1-180) (lanes 2 and 6), GST-AL1(119-352)(lanes 3 and 7), and GST-
 15 AL1(119-180) (lanes 4 and 8).

Figure 5A shows the interaction of mutant AL1 proteins with wild type AL1 in a yeast dihybrid assay. Expression cassettes for wild type AL1 fused to the GAL4 DNA binding domain and mutant AL1 fused to the GAL4 activation domain were co-transformed into yeast. Interactions between the wild type and mutant AL1 proteins were assayed by
 20 measuring B-galactosidase.

Figure 5B shows the interaction of mutant AL1 proteins with maize Rb in a yeast dihybrid assay. Expression cassettes for maize Rb (amino acids 214-866, Ach et al., *Mol. Cell. Biol.* 17:5077 (1997)) fused to the GAL4 binding domain and mutant AL1 fused to the GAL4 DNA activation domain were co-transformed into yeast. Interactions between the wild
 25 type and mutant AL1 proteins were assayed by measuring B-galactosidase.

Figure 6A shows that transient replication of TGMV B DNA is impaired by mutations in the AL1 oligomerization domain and upstream stabilizing region. Double-stranded DNA replication was analyzed in protoplasts co-transfected with expression cassettes for AL3, wild type or mutant AL1 proteins, and a TGMV B replicon. Total DNA
 30 was isolated three days post-transfection and analyzed by DNA gel blot hybridization using a radiolabeled TGMV B probe. Lanes correspond to transfections with wild type AL1 (lane 1), AL1 FQ118 (Ala13, lane 2), AL1 D120 (Ala14, lane 3), AL1 RS-R125 (Ala 1, lane 4), AL1 QT130 (Ala2, lane 5), AL1 ND133 (Ala3, lane 6), AL1 E--N140 (Ala4, lane 7), AL1

KEE146 (Ala5, lane 8), AL1 REK154 (Ala6, lane 9), AL1 EKY159 (Ala7, lane 10), AL1 Q-HN165 (Ala8, lane 11), AL1 N-DR172 (Ala9, lane 12), and AL1 K--E179 (Ala10, lane 13).

Figure 6B graphs the relative repression of the AL1 promoter in protoplasts transfected with an expression cassette for wild type AL1 or a mutant AL1, and a reporter with the AL1 promoter and luciferase reporter gene (*luc*).

Figure 7A shows that AL1 proteins defective for viral DNA replication interfere with TGMV A DNA replication. Protoplasts were co-transfected with 2 µg of a TGMV A replicon and 40 µg of expression cassettes coding for mutant AL1 proteins. Total DNA was isolated 3 days post-transfection and analyzed by DNA gel blot hybridization using a radiolabelled TGMV A probe. The top panel shows a representative blot of the replication interference assay. Lanes correspond to transfections of TGMV A DNA with an empty expression cassette (lane 1) and expression cassettes for AL1 FQ118 (Ala13, lane 2), AL1 D120 (Ala14, lane 3), AL1 RS-R125 (Ala1, lane 4), AL1 REK154 (Ala6, lane 5), AL1 EKY159 (Ala7, lane 6), AL1 Q-HIN165 (Ala8, lane 7), AL1 N-DR172 (Ala9, lane 8). The lower panel graphs the level of replicated viral DNA in the presence of excess mutant AL1 relative to wild type replication, averaged from at least three experiments.

Figure 7B shows results of experiments as described for **Figure 7A**, where the top panel is a representative blot of wild type replication interference by truncated AL1 proteins. Lanes correspond to transfections of TGMV A DNA with an empty expression cassette (lane 1), GST-AL1(119-180) (lane 2), AL1(1-180) (lane 3), AL1 N-DR172(134-352) (lane 4) and AL1 N-DR172 (lane 5). The bottom panel shows the levels of replicated viral DNA in the presence of excess mutant AL1 relative to wild type replication, averaged from at least three experiments.

Figure 8A shows a schematic of TGMV AL1. Solid boxes mark the location of the three motifs conserved among RCR initiator proteins, the oval indicates a predicted set of α -helices, and the stippled box shows the location of the ATP binding domain. The AL1 sequence between amino acids 132 to 156 (SEQ ID NO: 99) is shown, with the locations of the predicted α -helices 3 and 4 indicated. Vertical lines mark the positions of the alanine substitutions. Mutations are designated by the corresponding wild type sequence and the position of the last amino acid that was modified.

Figure 8B shows results of studies wherein an expression cassette, Zm214C, encoding amino acids 214 to the C-terminus of ZmRBR1, fused to the Gal4 DNA binding domain, was co-transformed into yeast with cassettes for either wild type or mutant AL1

fused to the Gal4 activation domain. Protein interactions were assayed by measuring β -galactosidase activity in total protein extracts and normalized to wild type (100). Filled bars indicate mutants strongly impaired for ZmRBR1 binding, hatched bars mark mutants moderately impaired in this activity, whereas open bars indicate mutants with activity similar to or greater than wild type AL1. The error bars correspond to two standard errors. The effects of the mutations on AL1-AL1 interactions (oligomerization activity) are indicated on the right.

Figure 9A shows that mutant L148 reduces replication and symptom severity. DNA replication of TGMV AL1 mutants was analyzed in tobacco protoplasts. Total DNA was isolated 72 hours post-transfection and analyzed by DNA gel blot hybridization using a radiolabeled TGMV A probe. Lanes 1-6 are transfections with TGMV A replicons with either wild type (lane 1) or mutant AL1 open reading frames corresponding to K144 (lane 2), EE146 (lane 3), A147Y (lane 4), L148 (lane 5), and II151 (lane 6). The positions of double (ds) and single (ss) stranded forms of TGMV A DNA are marked on the left. The relative accumulation of viral DNA is given at the bottom of each lane with wild type set at 100. No signal was detected in mutants A147Y and II151 even with longer exposures.

Figure 9B shows that *N. benthamiana* plants infected with the pRBR-binding mutant L148 developed chlorosis along the veins but no leaf curling and stunting characteristic of wild type TGMV infection. These milder symptoms were maintained over a 5-week infection period.

Figure 9C shows the results of *N. benthamiana* plants cobombarded with DNAs corresponding to TGMV A and B replicons. The AL1 open reading frames of the A components were either wild type (lane 1), or carried the K144 (lanes 2-4), EE146 (lanes 5-7), A147Y (lanes 8-10), L148 (lanes 11-13), or II151 (lanes 14-16) mutations. Total DNA (2.5 μ g/lane) was isolated from young leaves of three plants for each construct at 14 days post-infection and analyzed on DNA gel blots. Viral DNA was detected using a radiolabeled probe specific for TGMV A.

Figure 10 shows that the ZmRBR1-binding activity of AL1 is differentially affected by substitutions at position 148. The ZmRBR1 214C cassette fused to the Gal4 DNA binding domain was co-transformed into yeast with Gal4 activation domain cassettes for either wild type (L148) or mutant AL1 coding sequences (on the left) and analyzed as described in **Figure 8B**. The arrow indicates the relative α -helical tendency of each amino acid substitution (O'Neil et al., *Science* 250:646-651 (1990)). Filled, hatched and open bars are as

in **Figure 8**. The effect of the mutations on AL1 oligomerization activity is indicated on the right.

Figure 11A shows how RBR1 interacts with the replication proteins of CbLCV and TYLCV. The ZmRBR1 214C cassette fused to the Gal4 DNA binding domain was co-transformed into yeast with Gal4 activation domain cassettes for either TGMV AL1, TYLCV C1, CbLCV AL1 or mutant CbLCV AL1 CbL148 (on the left). Protein interactions were assayed by measuring β -galactosidase activity in total protein extracts and normalized to wild type TGMV AL1 (100). The error bars correspond to two standard errors.

Figure 11B shows the helix 4 motifs of TGMV AL1 (amino acids 144-156; **SEQ ID NO: 100**), TYLCV-DR C1 (amino acids 142-154; **SEQ ID NO: 101**) and CbLCV AL1 (amino acids 141-153; **SEQ ID NO: 102**). The conserved leucine residue in the helix center that was mutated to an alanine in CbL145 is marked with a dot. A consensus for begomovirus AL1/C1 proteins is shown at the bottom.

The present invention now will be described more fully herein.

Detailed Description of the Invention

As used herein, the terms “a,” “an” and “the” can mean one or more than one. For example, “a cell” can mean a single cell or a plurality of cells.

The present method utilizes the expression of trans dominant mutants of the geminivirus replication protein Rep, or AL1/C1, to confer increased resistance to geminiviruses in transgenic plants. While not wishing to be held to a single underlying theory, the present inventors hypothesize that the mutant proteins interfere with the replication activity of the wild type viral protein that is produced by the infecting geminiviruses, thus reducing the replication of infecting viruses and leading to enhanced resistance.

The present inventors determined that certain mutations in the AL1 sequence enhance the properties of the Rep protein that are useful in creating transgenic geminivirus resistant plants. The present invention provides nucleic acid constructs useful in producing transgenic plants with increased resistance to geminivirus infection. Prior to the present invention, trans dominant mutants of the Rep (AL1) protein contained mutations in catalytic motifs, e.g., the active site for DNA cleavage (motif III) and the ATPase domain (e.g., in the P-loop sequence).

Nucleic acids according to the present invention encode a mutant AL1 protein, wherein the protein acts as a trans dominant negative mutant, and can comprise a mutation in the Rb binding domain that decreases binding of the AL1 protein to the plant retinoblastoma protein, compared to that which would occur in the presence of wild type AL1. In TGMV virus, the Rb binding domain is found between amino acids 100 - 180. The present mutation Ala5 (EKY159) and Leu are examples of such mutations, resulting in decreased Rb binding compared to wild type AL1.

Nucleic acids according to the present invention encode a mutant AL1 protein, wherein the protein acts as a trans dominant negative mutant, and has a mutation in the oligomerization domain that decreases binding of the mutant AL1 to wild type AL1, compared to that which would occur in the presence of only wild type AL1. Such mutants result in decreased replication of infecting geminiviruses. The present mutation Ala5 (EKY159) and Leu are examples of such mutations, resulting in decreased AL1/AL1 binding compared to wild type AL1.

Nucleic acids according to the present invention encode a mutant AL1 protein, wherein the protein acts as a trans dominant negative mutant, and further comprises a mutation that results in enhanced repression of the viral promoter.

Specific examples of AL1 mutations in the TGMV viral genome are provided herein. It will be apparent to those skilled in the art that, in related geminiviruses, the retinoblastoma binding domain and oligomerization domain can be determined as shown herein. Mutations similar to the present TGMV mutations for other geminiviruses can thus be obtained.

The Geminiviridae family consists of three subgroups that differ with respect to insect vector, host range and genome structure. Subgroup I includes leafhopper-transmitted viruses that generally infect monocot plants and have single-component genomes. Subgroup III includes whitefly-transmitted viruses that infect dicot plants and most commonly have bipartite genomes. Subgroup II viruses are transmitted by leafhoppers and have single-component genomes like Subgroup I, but infect dicot plants like Subgroup III. Members of the three subgroups use similar replication and transcription strategies, although differences exist.

Geminiviruses have small genomes consisting of either one or two circular ss DNA molecules ranging from about 2.5 to about 3×10^3 nucleotides in size. The genomic DNAs contain divergent coding sequences separated by 5' intergenic regions. The coding capacity of the genomes varies among the different subgroups. Subgroup I viruses specify four open

reading frames for polypeptides greater than 10kDa, whereas subgroup II and III viruses encode six to seven open reading frames. There are currently two nomenclatures for geminivirus genes. The first nomenclature identifies viral genes as to whether they are specified by the virion (V) or complementary (C) sense DNA strands, whereas the second
5 nomenclature designates genes with respect to the left (L) or right (R) of the 5' intergenic region. The C and L designations are equivalent, as are the V and R designations.

The genomes of Subgroup III geminiviruses typically consist of two DNA components, designated A and B. Both components are required for efficient infection of host plants. The A component encodes all of the information necessary for viral replication
10 and encapsidation, whereas the B component cannot replicate in the absence of A DNA, but is required for systemic movement of the virus and symptom production in infected plants. The A component typically contains five open reading frames (ORFs), four of which (AL1/C1, AL2/C2, AL3/C3 and AL4/C4) are specified by overlapping sequences on the complementary strand. Mutations in the AL3 gene result in severely delayed and attenuated
15 symptoms (Morris et al., *J. Gen. Virol.* 72:1205 (1991); Etessami et al., *J. Gen. Virol.* 72:1005 (1991); Sung and Coutts, *J. Gen. Virol.* 76:1773 (1995)).

AL1/C1 (Rep) and AL3/C3 (Ren) proteins are involved in geminivirus replication, and AL1/C1 (Rep) and AL2/C2 proteins act in regulating viral gene expression. Mutation of the AL1 open reading frame was shown to block viral replication, whereas an AL3 mutant
20 resulted in reduced DNA levels (Sunter et al., *Virology* 179:69 (1990); Sung and Coutts, *J. Gen. Virol.* 76:1773 (1995)). Additionally, transgenic plants that contained the AL1 gene and constitutively expressed the Rep protein in the absence of AL3 supported replication of DNA B, demonstrating that Rep is sufficient for replication in the presence of host factors. (Hanley-Bowdoin et al., *Proc. Natl. Acad. Sci. USA* 87:1446 (1990); Elmer et al. *Nucleic*
25 *Acids Res.* 16:7043 (1988)).

The genomes of all geminiviruses employ the same general strategy for duplication and expression: a rolling circle replication system that amplifies ssDNA and produces dsDNAs that serve as templates for replication and transcription. The double-stranded form of DNA is divergently transcribed from a 5' intergenic region that also includes the plus-
30 strand origin of replication.

Rolling circle replication is a two-step process involving synthesis of the leading and lagging-strand DNA, which are separate events. The single-stranded 'plus' strand is first used as a template for the synthesis of the 'minus' strand, resulting in a double-stranded replicative

form (RF). The replicative form then serves as a template for plus-strand synthesis to generate free ssDNA. A site-specific nick primes plus-strand DNA synthesis (a hallmark of rolling circle replication systems). In subgroup I viruses, minus-strand synthesis appears to be primed by RNA that is most likely generated by pol α /primase complex. The mechanism of minus-strand replication in subgroup III is unknown. (The plus strand corresponds to the virion strand found in both ssDNA and dsDNA; the minus strand is the complementary strand found only in dsDNA).

Thus, geminivirus replication requires two origins, one for plus-strand synthesis and one for minus-strand synthesis. The plus-strand origin of geminiviruses from all three subgroups has been mapped to the 5' intergenic region, which also contains the promoters for virion and complementary-sense transcription. The *cis* elements that mediate viral replication and transcription are best characterized for the Subgroup III geminivirus, TGMV.

Geminiviruses fall into three subgroups based on their insect vector, host range and genome structure. Most dicot-infecting viruses have two genome components, designated A and B, and are transmitted by whiteflies. The single genome components of monopartite, dicot-infecting geminiviruses most resemble the A components of the bipartite viruses. The genome components are arranged similarly with 5' intergenic regions separating divergent transcription units. The 5' intergenic regions contain the viral replication origin (Revington et al., *Plant Cell* 1:985 (1989); Lazarowitz et al., *Plant Cell* 4:799 (1992)) and transcription signals (Eagle et al., *Plant Cell* 6:1157 (1994); Orozco et al., *Virology* 242:346 (1998)).

Dicot-infecting bipartite geminiviruses encode two replication proteins, AL1 and AL3, and recruit the remainder of their replication machinery from the host plant. For monopartite dicot-infecting geminiviruses such as tomato yellow leaf curl virus (TYLCV), the equivalent proteins are designated as C1 and C3, respectively. The AL1 (Rep) protein is the only viral protein essential for viral replication (Elmer et al., *Plant Mol. Biol.* 10:225 (1988); Hayes and Buck, *Nucleic Acids Res.* 17:10213 (1989); Hanley-Bowdoin et al., *Proc. Natl. Acad. Sci. USA* 87:1446 (1990)). AL1 induces the synthesis of host replication machinery in infected plant cells (Nagar et al., *Plant Cell* 7:705 (1995)). The AL3 protein is not required for replication, but enhances the level of viral DNA accumulation (Etessami et al., *J. Gen. Virol.* 72:1005 (1991); Morris et al., *J. Gen. Virol.* 72:1205 (1991)).

AL1 interferes with normal cell cycle regulation in plants and, subsequently, transgenes are silenced after several generations. Thus, AL1 proteins defective in multiple activities offer the best strategy for production of long term resistance.

The present invention is directed to the production of transgenic plants having increased resistance to geminivirus infection, to nucleic acid constructs useful in producing such plants, and to plant cells transformed with such constructs. The nucleic acid constructs contain a nucleotide sequence encoding a mutant AL1 protein or portion of a mutant AL1 protein.

Mutant AL1 proteins according to the present invention can comprise a mutation in the Rb binding domain, such that binding of AL1 to Rb in the plant cell is decreased. Rb is a negative regulator of the cell cycle and a common target of DNA viruses. Interference with the endogenous Rb protein leads to an uncontrolled cell cycle.

Mutant AL1 proteins according to the present invention can comprise a mutation in the oligomerization domain, to produce trans dominant negative mutants. Trans dominant negative mutant proteins negatively interfere in trans with geminiviral replication during infection.

Mutant AL1 proteins according to the present invention can comprise a mutation that enhances repression of the AL1 promoter. AL1 regulates viral DNA replication and transcriptional repression by binding to a conserved sequence in the overlapping plus-strand replication origin and the AL1 promoter. Mutants with enhanced repression of the AL1 promoter will interfere with replication of infecting wild-type geminiviruses.

Specific mutant Rep proteins of the present invention (see **Tables 1-3**) include proteins comprising the amino acid sequences shown as Leu (**SEQ ID NOs:75&76**), Ala1 (**SEQ ID NOs:55&56**), Ala4+5 (**SEQ ID NOs:77&78**), Ala6 (**SEQ ID NOs:65&66**), Ala7 (**SEQ ID NOs:67&68**), Ala8 (**SEQ ID NOs:69&70**), Ala9 (**SEQ ID NOs:71&72**), Ala13 (**SEQ ID NOs:51&52**) and Ala14 (**SEQ ID NOs:53&54**); these mutants are modified in the oligomerization domain and interfere with viral replication. They also display the property of enhanced repression of transcription from the Rep promoter. Because of their enhanced repression activity, the oligomerization mutants are likely to confer enhanced geminivirus resistance to transgenic plants. Mutants Ala13, Ala4+5, Ala6, and Ala7 also display impaired Rb binding and thus can be stably expressed in plants. The present invention additionally provides mutant Rep proteins shown as Ala2 (**SEQ ID NOs:57&58**), Ala3 (**SEQ ID NOs:59&60**), Ala4 (**SEQ ID NOs:61&62**), Ala5 (**SEQ ID NOs:63&64**) and Ala10 (**SEQ ID NOs:73&74**).

The present invention further provides the mutant proteins listed in **Tables 4 and 5**, comprising the amino acid sequences shown as K144 (**SEQ ID NOs:79&80**), E145 (**SEQ ID**

NOs:81&82), E146 (SEQ ID NOs:83&84), EE146 (SEQ ID NOs:85&86), A147Y (SEQ ID NOs:87&88), L148 (SEQ ID NOs:89&90), L148V/L148V* (SEQ ID NOs:91&92 and 93&94), L148G (SEQ ID NOs:95&96), L145A (SEQ ID NOs:111&112) and II151 (SEQ ID NOs:97&98). The present invention provides a mutant AL1 protein comprising the amino acid sequence of any of the mutants described herein. Such a mutant AL1 protein will have the amino acid sequence of the wild type AL1 protein (SEQ ID NO:1 for TGMV; SEQ ID NO:107 for CbLCV), which is available, for example in the Genbank database (and is incorporated herein in its entirety by reference), including the mutation as shown in the amino acid sequence of the respective mutants described herein in lieu of the wild type sequence at the mutated site. Further provided in this invention is a nucleic acid encoding the mutant AL1 protein of this invention. The nucleic acid sequence of this invention can be any sequence that encodes the mutant AL1 protein described herein and encompasses a variety of coding sequences due to the degeneracy of the nucleic acid code. For example, a nucleic acid encoding a mutant AL1 protein of this invention can have the nucleic acid sequence of wild type AL1 protein (SEQ ID NO:50 for TGMV; SEQ ID NO:110 for CbLCV) as is available, for example, from the Genbank database (and is incorporated by reference herein in its entirety), and including the mutation as shown in the nucleic acid sequence of the mutant AL1 protein as described herein in lieu of the wild type sequence at the mutated site.

Accordingly, mutant AL1 proteins of the present invention can comprise any combination of (1) a mutation in the oligomerization domain (trans dominant negative mutant); (2) a mutation in the Rb binding domain (decrease binding of AL1 to Rb); and/or (3) a mutation that increases repression of the AL1 promoter.

Mutant AL1 proteins of the present invention can comprise or further comprise (in combination with another mutation as described herein) a mutation in the DNA cleavage and/or ATPase domain, where such mutations produce trans dominant negative mutants.

The present methods can thus utilize expression of a (mutant) trans dominant viral AL1/C1 protein in transgenic plants, where the mutant contains a change in the Rb binding domain that decreases binding of the Rep protein to the Retinoblastoma homologue found in the plant to be transformed (compared to binding of the Rb homologue to wild-type AL1 protein). Mutant AL1/C1 proteins can also have a mutation in the oligomerization domain, such that viral replication is suppressed (compared to that which would occur in the presence of wild type AL1 protein). The combination of a mutation in the Rb domain as described

above, and a mutation in the oligomerization domain results in a stably expressed AL1 (Rep) protein capable of suppressing the replication of infecting geminivirus.

The development of geminivirus resistant plants is desirable. One approach is the expression of trans dominant negative mutants of the geminivirus protein Rep (also called AL1 or CI), which is the only viral protein required for geminivirus replication.

The geminivirus AL1 protein, which localizes to the nuclei of infected plant cells, plays key roles in geminivirus DNA replication and transcription. AL1 confers virus-specific recognition of its cognate origin of replication and initiates plus-strand DNA synthesis. AL1 also functions as a transcriptional repressor by blocking host-mediated activation of its promoter. Biochemical studies of TGMV AL1 have established that it is a sequence-specific DNA binding protein, a DNA cleavage/ligation enzyme and an ATPase. AL1 also forms large oligomers and interacts with the replication enhancer protein, AL3. All of the known activities of AL1 except for ATP hydrolysis are mediated by overlapping domains in the N-terminus of the protein.

The present invention is directed to a series of AL1 mutants modified in conserved sequence and structural domains of the protein (Orozco et al., *J. Biol. Chem.* 273:24448 (1998)). Analysis of the mutant proteins established the importance of these motifs for AL1 function and revealed that DNA binding and cleavage are tightly linked, involving many of the same amino acids. However, the domain requirements for these activities can be distinguished by their dependence on AL1/AL1 interactions, which are only required for DNA binding and not for DNA cleavage/ligation.

AL1 also acts as a plant cell regulator to create an environment that facilitates efficient viral replication. Analysis of transgenic plants that constitutively expressed the TGMV viral replication protein revealed that AL1 induces the expression of a host DNA synthesis protein, proliferating cell nuclear antigen (PCNA), in mature cells. This observation suggests that AL1 is analogous to the DNA tumor antigen proteins of mammalian viruses, which induce replication machinery in their hosts by altering cell cycle and transcriptional controls. The elevated levels of PCNA mRNA detected in TGMV infected tissue and the interaction between TGMV AL1 and a plant homologue of the animal tumor suppressor protein, retinoblastoma, suggest that AL1 can use similar strategies to modify plant cells.

The present invention utilizes mutant Rep proteins that are impaired for interaction with the retinoblastoma (RB) homologues from maize and *Arabidopsis*. Rb is a negative regulator of the cell cycle and a common target of DNA tumor viruses in mammalian cells. A

Rep mutant that cannot bind Rb is likely to prove less detrimental to plants, and thus could be combined with other mutations for stable expression of trans dominant negative Rep mutants in transgenic plants.

The present invention provides Rep mutations suitable for use in transgenic plants.

5 All of the mutations target one or more highly conserved residues in geminivirus Subgroup II and III Rep proteins.

10 The use of Rep mutants that are modified in the DNA cleavage or ATPase domains to produce transgenic virus resistant plants has been problematic, with a lack of stable expression of Rep in the transgenic plants. The use of Rep mutants according to the present invention, such as one combining an oligomerization mutation with a retinoblastoma binding mutation, overcomes this difficulty. The present mutants Ala4+5 and Ala7 display impaired Rb binding, indicating stable expression in plants. The Leu, Ala4+5, Ala6, Ala7, Ala8, Ala9, Ala13 and Ala14 mutants act as trans dominant negative mutants in transient replication assays, and display enhanced repression activity in transcription assays.

15 Together, the Ala1, Ala4+5, Ala6, Ala7 and Ala13 mutations define a novel Rb binding domain found in the Rep proteins of Subgroup II and III geminiviruses. The Rb binding motif has not been identified before. The Ala1 and Ala13 mutations are located in a 17 amino acid sequence that is highly conserved among most dicot-infecting geminiviruses. The Ala4+5 and Ala6 mutations are in a strongly predicted helix-loop-helix structural motif
20 found in the Rep proteins of all geminiviruses. Hence, these mutations are likely to be broadly applicable for developing resistance strategies against all subgroups of geminiviruses. The Ala1, Ala 4+5, Ala6, and/or Ala7 mutations can be incorporated into a Rep trans dominant mutant to stabilize Rep expression. The interaction of a geminivirus Rep protein with a retinoblastoma homologue from a dicot species has not been described before.

25 The Ala4+5, Ala6, Ala7 and Ala13 mutations can be sufficient to confer stable geminivirus resistance to plants because they also display trans dominant negative interfering activity in replication assays and enhanced repression activity in transcription assays.

The Ala1, Ala4+5, Ala6, Ala7, Ala13 and LEU mutants are TGMV Rep mutants containing amino acid mutations in the sequence of TGMV Rep from amino acids 111-180
30 (TLVWGEFQVD GRSARGGCQT SNDAAAEALN ASSKEEALQI IREKIPEKYL FQFHINLNSNL DRIFDKTPEP (SEQ ID NO: 2)), where the underlined amino acids correspond to a conserved helix-loop-helix motif found in the Rep proteins of all geminiviruses.

AL1 is a large oligomeric protein that binds double-stranded DNA, catalyzes cleavage and ligation of single-stranded DNA, and interacts with other viral and host proteins. Earlier experiments mapped the TGMV AL1 oligomerization domain between amino acids 121–181 and the DNA binding domain within amino acids 1 to 181. In the studies reported herein, truncated TGMV AL1 proteins were used to refine the limits of the oligomerization domain and generate site-directed mutations in conserved charged hydrophobic residues of the domain to assess their importance in AL1 function. Nearly all of the mutants attenuated or abolished AL1-directed viral DNA replication. In contrast, the replication defective mutants were enhanced for AL1-mediated transcriptional repression. Yeast two-hybrid experiments revealed that several of the mutations reduced AL1 complex stability, particularly amino acids 157-159. This mutation also disrupted protein interactions in insect cells between the full-length mutant AL1 and the AL1 oligomerization domain fused to GST. In addition, replication defective AL1 mutants interfered with DNA synthesis from wild type TGMV A, indicating that these proteins are good candidates for use in developing geminivirus resistant transgenic plants.

AL1 has several functions in replication and transcription. AL1 mediates both virus-specific recognition of its cognate origin, and transcriptional repression by binding to the direct repeat sequence. Geminivirus replication and termination is regulated by AL1 DNA cleavage and ligation within an invariant sequence in the loop of a conserved hairpin. In addition, AL1 induces accumulation of a host replication factor, PCNA, in infected cells. Recombinant AL1 specifically binds double-stranded DNA, cleaves and ligates single-stranded DNA, and hydrolyzes ATP. Geminivirus AL1 also interacts with itself, AL3 and a plant homologue of the mammalian retinoblastoma protein, PRB1.

The domains of TGMV AL1 for double stranded DNA binding, single stranded DNA cleavage and ligation, and AL1 oligomerization have previously been mapped (Orozco et al., *J. Biol. Chem.* 272:9840 (1997); Orozco et al., *J. Biol. Chem.* 273:24448 (1998)). The AL1 DNA cleavage and ligation domain is located in the first 120 amino acids and the oligomerization domain maps between amino acids 120-181. DNA binding activity requires amino acids 1-130 for protein-DNA contacts and the AL1 oligomerization domain. The Examples provided herein describe truncation studies and use of site-directed mutants to show that the oligomerization domain lies between amino acids 134 and 181 and that additional sequences outside the core domain influenced protein interactions. The mutations were also tested for their impact on viral DNA replication and transcription (in protoplasts).

Yeast dihybrid assays established that Leu, Ala1, Ala4+5, Ala6, Ala7 and Ala13 Rep mutants are impaired for interaction with the Rb homologues from maize and/or *Arabidopsis*. Leu, Ala4+5, Ala6 and Ala7 were shown to act as trans dominant negative inhibitors of geminivirus replication and displayed enhanced transcriptional repression activity in *Nicotiana*. These activities in combination with impaired Rb binding make Leu, Ala4+5, Ala6 and Ala7 excellent candidates for conferring geminivirus resistance to plants.

TGMV AL1 regulates viral DNA replication and transcriptional repression by binding to a conserved sequence in the overlapping plus-strand replication origin and AL1 promoter. Previous experiments showed that AL1/DNA interactions require both the DNA binding domain (amino acids 1-131) and oligomerization domain (amino acids 121-181) of AL1. To further map the oligomerization domain, AL1 truncations of TGMV AL1 (SEQ ID NO: 1) were generated that deleted additional amino acid sequences from the N- and C-terminus. Truncating an additional 13 amino acids from the C-terminus abolished AL1 interactions, demonstrating that the C-terminal boundary of the oligomerization domain is between amino acids 168 and 181. The N-terminal boundary of the oligomerization domain was unclear when interactions were observed with the full length GST-AL1 protein. However, AL1 oligomerized with a GST-AL1 fusion protein containing AL1 amino acids 119-180, demonstrating that this region is sufficient to form AL1 complexes. When N-terminal truncations were co-purified with GST-AL1 119-180, protein interactions were lost when amino acids 134-147 were removed. Thus, the N-terminal boundary of the minimal oligomerization domain lies between position 134 and 147, which includes two highly predicted alpha-helices. In the presence of full-length GST-AL1, deletion of the helices resulted in near background levels of interactions, indicating that weak interactions can occur in the region 147 to 180 and that the helices are important for stable complex formation.

Alanine substitutions (see **Figure 1** and **Table 3**) were generated at highly conserved, charged or hydrophobic residues across the oligomerization domain to assess the contributions of specific amino acids *in vitro* and *in vivo*. The mutant proteins were first analyzed in insect cells for their effect on AL1 oligomerization. Although the amino acid changes were insufficient to disrupt interactions with the full-length GST-AL1, a more stringent assay using GST-AL1 119-180 revealed that AL1 EKY159 (Ala7) was impaired for oligomerization. However, AL1 EKY159 (Ala7) interacted with an AL1 truncation lacking the N-terminus (or, including the C-terminus) that retained the core oligomerization domain, suggesting that multiple interfaces stabilized the protein complexes. These results are

consistent with the observation that additional contacts outside the core oligomerization domain contributed to interactions with wild type truncated AL1.

A yeast two-hybrid assay was employed to determine the quantitative effects of the mutations on AL1 oligomerization with the wild type protein. Consistent with the results from insect cells, AL1159 (Ala7) was the most impaired for protein interactions. Mutations that significantly altered AL1 interactions in yeast were clustered between amino acids 152 and 172, within the region defined as the minimal oligomerization domain by the truncation experiments. Mutations in the helices slightly impaired AL1 interactions compared to the 152 to 172 region, consistent with the deletion analysis, suggesting that this region is also required. The alanine substitutions are not predicted to disrupt helix formation although the sequence of the helices is fairly divergent among AL1 proteins. These results indicate that the sequence is an important feature of this region. Alanine substitutions adjacent to the oligomerization domain (D120 (Ala14) and RS-R125 (Ala1)) indicate that this region contributes contacts that stabilize protein interactions.

AL1 subunits defective for viral DNA replication can potentially form complexes with wild type AL1 and interfere with normal viral DNA replication. Thus, expression vectors for seven mutant proteins were co-transfected into protoplasts with a wild type TGMV replicon. All of the mutants significantly reduced single stranded and double stranded DNA synthesis. Mutations in the conserved ATP binding site and DNA cleavage motif and a C-terminal truncation of AL1 from related geminiviruses were previously shown to interfere with replication in transgenic plants. All of these mutant proteins are candidates for developing plants resistant to geminivirus infection. However, AL1 toxicity to plant cells results in silencing of transgenes over successive generations. In addition, single mutations can potentially recombine with wild type virus to produce a functional protein. The goal of the present invention is to design non-toxic mutants of AL1 that are defective in multiple functions.

Ten of the twelve mutants enhanced repression of the AL1 promoter 2- to 4- fold above wild type AL1. AL1 K--E179 is essentially wild type for replication and AL1 oligomerization. AL1RS-R125 lacks DNA binding activity that is essential for AL1-mediated repression. No specific correlation was observed between the strength of AL1 interactions in yeast and the differential effect of the mutations on replication versus transcriptional repression. For example, AL1 EKY159 (Ala7) and AL1 Q-HN165 (Ala8) altered AL1 interactions to 0% and 102% of wild type, respectively, but displayed essentially identical

effects on replication and transcription. Similarly, AL1 ND133 (Ala3) repressed expression nearly 4-fold higher than wild type AL1 and greatly reduced replication, whereas protein interactions were comparable to wild type AL1. This suggests the effect of the mutations can be attributed to changes in the structure and/or the number of AL1 subunits in the complex.

- 5 Thus, structural analysis of the wild type and mutant AL1 proteins will be essential to determine the cause of the phenotypic changes observed with the mutant proteins. Ten of the mutations showed increased transcriptional repression concomitant with decreased viral replication.

10 The present methods utilize nucleic acid constructs encoding mutated versions of naturally occurring geminivirus AL1/C1 (Rep) proteins. The term "mutated" as used herein regarding proteins or polypeptides means that at least one amino acid in the wild-type or naturally occurring protein or polypeptide sequence has been replaced with a different amino acid, and/or added and/or deleted from the sequence. Preferably at least two or more adjacent amino acids in the wild-type sequence are replaced, added and/or deleted. Mutant AL1/C1
15 proteins can contain from about 2 to about 30, or more, replaced, added and/or deleted amino acids. A particularly preferred mutation is the replacement of conserved, charged or hydrophobic amino acid residues with alanine.

As used herein, the term "AL1/C1" or "Rep" protein refers to the geminivirus proteins that are known in the art as AL1/C1 proteins in geminiviruses. Subgroup II and III
20 geminiviruses encode a protein that is identifiable by those skilled in the art, based on structure and/or function, as the AL1/C1 protein. As used herein, the term "AL1/C1" as it is applied to polypeptides includes fragments of AL1/C1 proteins. As used herein, the term "AL1/C1" as it is applied to nucleic acid sequences (including naturally occurring sequences and genes, and synthesized nucleic acid sequences) refers to sequences that encode a
25 naturally occurring AL1/C1 protein or polypeptide, or a mutated AL1/C1 protein or peptide as described herein.

Mutated AL1/C1 proteins and polypeptides useful in the present methods are those which, when expressed in a plant cell, reduce the sensitivity of the cell (or a plant comprising such cells) to infection by a geminivirus. Mutated AL1/C1 proteins and polypeptides useful
30 in the present methods are also those which, when expressed in a plant cell, increase or enhance the resistance or tolerance of the cell (or a plant comprising such cells) to infection by a geminivirus.

As used herein, "sensitivity" of a plant to infection by a geminivirus refers to the rate at which symptoms of geminivirus infection develop, and the severity of symptoms. Plants with reduced sensitivity to infection have delayed development of symptoms and/or less severe symptoms of geminivirus infection compared to that which occurs in a control plant.

5 As used herein, "tolerance" refers to plants that are infected with and contain a geminivirus, but do not show symptoms associated with viral infection. Tolerant crop plants are able to produce a good crop despite geminivirus infection. As used herein, plants that are "immune to infection" by a geminivirus are those in which replication of the virus is prevented. As used herein, plants that are "resistant" to infection by a geminivirus are those
10 that show both immunity to infection and tolerance.

It will be apparent to those skilled in the art that the ability of a plant to survive and thrive when exposed to geminiviruses is a continuum, from plants that are less sensitive to infection, to those that are tolerant to infection, to those that are resistant to, geminiviruses. A plant that shows enhanced resistance or tolerance to geminivirus infection is considered
15 herein to also show reduced sensitivity to geminivirus infection. In each case, the severity and/or rate of development of symptoms in plants with enhanced resistance (reduced sensitivity) to geminiviruses is less than that which occurs in a control plant.

Sensitivity, tolerance or resistance to geminivirus infection can be measured at the level of a plant cell or at the level of a single plant (e.g., by assessing the severity or rapidity
20 of symptom development), or at the level of a plurality of plants (e.g., by assessing the prevalence and/or severity of infection, or the crop yield). Sensitivity in transgenic plants can be assessed by comparison to non-transformed control plants of the same species.

As used herein, the terms "protein" and "polypeptide" are used interchangeably, and refer to a polymer of amino acids (dipeptide or greater) linked through peptide bonds. Thus,
25 the term "polypeptide" includes proteins, oligopeptides, protein fragments, protein analogs and the like. The term "polypeptide" contemplates polypeptides as defined above that are encoded by nucleic acids, are recombinantly produced, are isolated from an appropriate source, or are synthesized.

The mutated geminivirus AL1/C1 proteins useful in the present methods can be based
30 on any naturally occurring AL1/C1 protein. A series of site-directed mutants of any AL1/C1 protein can be prepared and screened for the ability to enhance geminivirus resistance (for example, using tobacco protoplast complementation assays as described in the Examples, below). Mutant AL1/C1 proteins or polypeptides capable of reducing sensitivity to

geminivirus infection in transgenic plants according to the methods herein are identified, and nucleic acid constructs capable of encoding the mutant protein are prepared according to methods known in the art, for use in producing transgenic plants with reduced sensitivity to (or increased resistance or tolerance to) geminivirus infection.

5 As used herein, a "native" or "naturally-occurring" nucleic acid sequence is a sequence that is found in non-transgenic cells or tissue. Native nucleic acid sequences are thus those that have not been artificially altered, such as by site-directed mutagenesis. Once native nucleic acid sequences have been determined, molecules having these sequences can be synthesized or produced using recombinant nucleic acid procedures as are known in the
10 art. As used herein, a native geminivirus nucleic acid sequence is that which can be isolated from or identified in a naturally occurring geminivirus.

Mutants of AL1/C1 proteins from various geminiviruses are suitable for use in the present invention, including but not limited to: Tomato golden mosaic virus (TGMV), tomato mottle virus, tomato yellow leaf curl virus (TYLCV), tomato leaf curl virus (TLCV), potato
15 yellow mosaic virus (PYMV), African cassava mosaic virus (ACMV), Indian cassava mosaic virus, bean golden mosaic virus (BGMV), bean dwarf mosaic virus, squash leaf curl virus, cotton leaf curl virus (CLCV), beet curly top virus (BCTV), Texas pepper virus and Pepper Huasteco virus. A preferred mutant protein or polypeptide is one in which one or more amino acid residues are replaced with alanine.

20 The sequences of AL1/C1 proteins from various geminiviruses are known and available through the GenBank database.

Unless otherwise stated, nucleotide sequences are presented herein by single strand only, in the 5' to 3' direction, from left to right. Nucleotides are represented herein in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission.

25 The methods and constructs of the present invention are useful in transforming dicot plant species to produce plants with reduced sensitivity to geminivirus infection. Dicots suitable for use in practicing the present invention include plants from the Fabaceae, Solanaceae, Brassicaceae, Rosaceae and Compositae families. Examples of plant species suitable for transformation with the DNA constructs of the present invention include but are
30 not limited to tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), soybean (*glycine max*), tomato (*Lycopersicon esculentum*), cassava (*Manihot esculenta*), beets, peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), citrus trees (*Citrus* spp.), corn or maize (*Zea mays*), beans (e.g., green beans (*Phaseolus vulgaris*) and lima beans (*Phaseolus*

limensis)), peas (*Lathyrus* spp.), sugarbeet, sunflower, carrot, celery, flax, cabbage and other cruciferous plants, pepper, strawberry, lettuce, alfalfa, oat, wheat, rye, rice, barley, sorghum and canola. Thus an illustrative category of plants which can be transformed with the constructs of the present invention are members of the family Solanaceae, and particular plants which can be transformed using the constructs of the present invention are tomato and tobacco.

A variety of techniques are available in the art for introduction of DNA constructs into a plant cell host. These include, but are not limited to, *Agrobacterium*-mediated transfection, injection, electroporation and microparticle bombardment. In preferred embodiments, plants are transfected using *Agrobacterium*-mediated transfection, or intact plants are inoculated using microprojectiles carrying a nucleic acid construct according to the present invention.

In practice, a crop comprising a plurality of plants of the invention can be planted together in an agricultural field. By "agricultural field" is meant a common plot of soil or a greenhouse. Thus, the present invention provides a method of providing a crop of transgenic plants.

Those familiar with recombinant DNA methods available in the art will recognize that one can employ a nucleic acid sequence coding for a mutant AL1/C1 protein of the present invention, joined in the sense orientation with appropriate operably linked regulatory sequences, to construct transgenic cells and plants. Appropriate regulatory sequences for expression of nucleic acid sequences in the sense orientation include any of the known eukaryotic translation start sequences, in addition to promoter and polyadenylation/transcription termination sequences.

Nucleic acid constructs (or "transcription cassettes") of the present invention include, 5' to 3' in the direction of transcription, a promoter as discussed above and, operatively associated with the promoter, a nucleic acid sequence encoding a mutant AL1/C1 protein of the present invention. The construct can optionally contain a termination sequence including stop signal for RNA polymerase. Each of these regulatory regions should be capable of operating in the cells of the tissue to be transformed. Any suitable termination signal can be employed in carrying out the present invention, examples thereof including, but not limited to, the *nos* terminator, the CaMV terminator, or native termination signals derived from the same gene as the transcriptional initiation region or derived from a different gene. The term "operatively associated," as used herein, refers to nucleic acid sequences on a single

nucleic acid molecule, which sequences are associated so that the function of one is affected by the other. Thus, a promoter is operatively associated with a nucleic acid sequence when it is capable of affecting the transcription of that sequence (i.e., the sequence is under the transcriptional control of the promoter). The promoter is said to be "upstream" from the sequence, which is in turn said to be "downstream" from the promoter.

The various fragments comprising the various constructs, transcription cassettes, markers, and the like can be introduced consecutively by restriction enzyme cleavage of an appropriate replication system, and insertion of the particular construct or fragment into the available site. After ligation and cloning, the nucleic acid construct can be isolated for further manipulation. All of these techniques are amply exemplified in the literature (see, e.g., J. Sambrook et al., Molecular Cloning, A Laboratory Manual (2d Ed. 1989) (Cold Spring Harbor Laboratory)).

The term "nucleic acid sequence" as used herein refers to a DNA or RNA molecule, and more particularly a linear series of deoxyribonucleotides or ribonucleotides connected to one another by bonds, typically phosphodiester bonds, between the 3' and 5' carbon of the adjacent pentoses.

The term "promoter" refers to a region of a DNA sequence that incorporates the necessary signals for the efficient expression of a coding sequence. This can include sequences to which an RNA polymerase binds but is not limited thereto, and can include other sequences to which other regulatory proteins bind, together with regions involved in the control of protein translation. Promoters employed in carrying out the present invention can be promoters that are constitutively active in the subject plant cell. Numerous constitutively active promoters that are operable in plants are available. A preferred example is the 35S promoter from fig wort mosaic virus (FMV), or the Cauliflower Mosaic Virus (CaMV) 35S promoter. In the alternative, the promoter can be promoter that is spatially active or active only in a specific tissue of the plant (see e.g., US Patent No. 5,459,252 for root-specific promoters), or an inducible promoter (e.g., a promoter active in plants that is induced by specific conditions, such as wounding or infection by specific pathogens).

Methods of making transgenic (or 'recombinant') plants of the present invention, in general, involve first providing a plant cell capable of regeneration (the plant cell typically residing in a tissue capable of regeneration). The plant cell is then transformed with a DNA construct comprising a transcription cassette of the present invention (as described herein) and a transgenic plant is regenerated from the transformed plant cell. The transforming step

can be carried out by any suitable technique as is known in the art, including but not limited to bombarding the plant cell with microparticles carrying the transcription cassette, infecting the cell with *Agrobacterium tumefaciens* containing a Ti plasmid carrying the transcription cassette, or any other suitable technique.

5 Vectors which can be used to transform plant tissue with the nucleic acid constructs of the present invention include both *Agrobacterium* vectors and ballistic vectors, as well as other suitable vectors known to those in the art. *Agrobacterium tumefaciens* cells containing a nucleic acid construct of the present invention are useful in methods of making transformed plants. Plant cells are infected with *Agrobacterium tumefaciens* to produce a transformed
10 plant cell, and then a plant is regenerated from the transformed plant cell, according to methods known in the art. Numerous *Agrobacterium* vector systems useful in carrying out the present invention are known (*see, e.g.*, U.S. Patent No. 4,459,355; U.S. Patent No. 4,795,855; U.S. Patent No. 4,940,838, the entire contents of each of which are incorporated by reference herein).

15 Microparticles carrying constructs of the present invention, which microparticle is suitable for the ballistic transformation of a plant cell, are also useful for making transformed plants of the present invention. The microparticle is propelled into a plant cell to produce a transformed plant cell and a plant is regenerated from the transformed plant cell. Any suitable ballistic cell transformation methodology and apparatus can be used in practicing the
20 present invention. Exemplary apparatus and procedures are disclosed in Sanford and Wolf, U.S. Patent No. 4,945,050; in Christou et al., US Patent No. 5,015,58; and in Agracetus European Patent Application Publication No. 0 270 356, entitled "Pollen-mediated Plant Transformation." (the entire contents of each of which are incorporated by reference herein).

25 Plant species can be transformed with the nucleic acid constructs of the present invention by the DNA-mediated transformation of plant cell protoplasts and subsequent regeneration of the plant from the transformed protoplasts in accordance with procedures known in the art. Fusion of tobacco protoplasts with DNA-containing liposomes or via electroporation is known in the art (Shilleto et al., *Methods in Enzymology*, 153:313-336 (1987)).

30 As used herein, transformation refers to the introduction of exogenous nucleic acid molecules into cells, so as to produce transgenic cells stably transformed with the exogenous nucleic acid. Transformed plant cells are induced to regenerate intact plants through application of cell and tissue culture techniques that are known in the art. The method of

plant regeneration is chosen so as to be compatible with the method of transformation. The stable presence and orientation of the exogenous DNA in transgenic plants can be verified by the Mendelian inheritance of the DNA sequence, as revealed by standard methods of DNA analysis applied to progeny resulting from controlled crosses.

5 Any plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, can be transformed with the constructs of the present invention. The term "organogenesis," as used herein, means a process by which shoots and roots are developed sequentially from meristematic centers; the term "embryogenesis," as used herein, means a process by which shoots and roots develop together in a concerted
10 fashion (not sequentially), whether from somatic cells or gametes. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristems, axillary buds, and root meristems), and induced
15 meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

Transgenic plants of the present invention can take a variety of forms. The plants can be chimeras of transformed cells and non-transformed cells; the plants can be clonal transformants (e.g., all cells transformed to contain the transcription cassette); the plants can comprise grafts of transformed and non-transformed tissues. The transformed plants can be
20 propagated by a variety of means known in the art, such as by clonal propagation or by classical breeding techniques.

The examples which follow are set forth to illustrate the present invention, and are not to be construed as limiting thereof.

EXAMPLE 1

Mutagenesis and Cloning of AL1 proteins: The plasmid pNSB148 containing the AL1 coding sequence in a pUC 118-background, was used as the template for site directed mutagenesis. The oligonucleotide primers and resulting clones are listed in **Table 4**. DNA fragments containing the mutations were verified by DNA sequence analysis. Plant expression cassettes for mutant AL1 proteins were generated by subcloning *Sall/NcoI* fragments from the mutant clones into the same sites in a wild type AL1 plant expression cassette pMON1549 (Fontes et al., *Plant Cell* 6:405 (1994); Fontes et al., *J. Biol. Chem.* 269:8459 (1994)). In pMON1549, AL1 expression is under the control of the cauliflower mosaic virus 35S promoter with a duplicated enhancer region and the E9 3' end.

Baculovirus vectors were generated for expressing mutant and truncated AL1 proteins in insect cells. Expression vectors coding for mutant AL1 proteins were generated by subcloning *BglII/BamHI* inserts from the mutant plant expression cassettes into the *BamHI* site of pMON27025 (Luckow et al, *J. Virol.* 67:4566 (1993)). Expression vectors for the truncated proteins AL1¹¹⁹⁻³⁵² (pNSB516), AL1¹⁻¹²⁰ (pNSB388), and AL1¹⁻¹⁸⁰ (pNSB517) have been described previously (Orozco et al., *J. Biol. Chem.* 272:9840 (1997)). N-terminal truncations, AL1¹³⁴⁻³⁵² and AL1¹⁴⁷⁻³⁵² were generated by inserting a DNA linker containing a start codon into the *NotI* site of pNSB593 and pNSB595. AL1¹⁶⁰⁻³⁵² was created by inserting an *SphI* linker into the *SspI* site of pMON1539. *SphI/BamHI* fragments from the resulting clones were inserted into the same sites of the baculovirus vector, pNSB448, to give pNSB803 (AL1¹³⁴⁻³⁵²), pNSB876 (AL1¹⁴⁷⁻³⁵²) and pNSB633 (AL1¹⁶⁰⁻³⁵²). The C-terminal truncation AL1¹⁻¹⁵⁸ (pNSB646) was created by digesting pMON1539 with *NdeI* and *SspI*, repairing with Klenow, and subcloning into the filled *BamHI* site of pMON27025. The AL1¹⁻¹⁶⁸ truncation, pNSB708, was created by inserting an *XbaI* linker into the repaired *BssHII* of pNSB609.

Yeast expression cassettes were generated containing the coding sequence for AL1 fused to the Gal4 DNA binding domain, pNSB736, or Gal4 activation domain, pNSB809. The pAS2-1 Gal4 BD and Gal4 AD cloning vectors were purchased from Clontech. pNSB736- The *BamHI/NdeI* fragment of pMON1539 was cloned into the same sites of pAS2-1 pACT2-Gal4 AD vector to give pNSB736. pNSB735 - the *BamHI/NdeI* fragment of pMon 1539 was cloned into the *SmaI* site of pACT2. pNSB809 replaced *AatII/BamHI* fragment

from pNSB735 with the *AatII/BamHI* fragment from pMON1549. Mutant AL1 expression cassettes were created by replacing the *AatII/BamHI* wild type fragments from pNSB735 with *AatII/BamHI* inserts from the mutant plant expression cassettes.

Transient Replication Assays: Protoplasts were isolated from *Nicotiana tabacum* NT suspension cells, electroporated and cultured according to published methods (Fontes et al., *J. Biol. Chem.* 269:8459 (1994)). The transfections contained 15µg each of replicon DNA containing a partial tandem copy of TGMV B (pTG1.4B described in Fontes et al., *Plant Cell*, 6:405 (1994)), wild type or mutant AL1 plant expression cassette and an AL3 plant expression cassette, (pNSB41 described in Fontes et al., *J. Biol. Chem.* 269:8459 (1994)).

Interference Assays: For the interference assays, transfections containing 2 µg of replicon DNA containing a partial tandem copy of TGMV A and 40 µg of mutant AL1 expression cassette or the empty expression vector. Total DNA was extracted 3 days post-transfection and analyzed for double- and single-stranded viral DNA accumulation by DNA gel blot hybridization.

Repression Assays: Protoplasts were isolated from *Nicotiana benthamiana* suspension cells, electroporated and cultured according to published methods (Eagle et al., *Plant Cell* 6:1157 (1994)).

AL1 Interactions in Yeast: The yeast strain Y187 was co-transformed with an expression cassette for wild type AL1 or maize Rb fused to the Gal4 binding domain (BD) and expression cassettes for mutant AL1 proteins fused to the Gal4 activation domain (AD). Total extracts were assayed for B-galactosidase activity using the substrate o-nitrophenyl B-D-galactopyranoside, essentially as described by Clontech (Palo Alto, CA). Protein concentrations were measured by Bradford assays. The activities were standardized against wild type AL1 fused to both the Gal4 activation domain and DNA binding domain.

EXAMPLE 2

The domains for TGMV AL1 DNA binding and DNA cleavage/ligation activity have been well defined and key structural and sequence motifs have been identified for these activities (Orozco et al., *J. Biol. Chem.* 273:24448 (1998)). In contrast, TGMV AL1 protein interactions with the viral protein AL3, plant retinoblastoma (Rb) homologue, and AL1 itself have been broadly mapped to overlapping domains in the center of the protein. In the present studies, additional N- and C-terminal truncations were generated to further define the limits of the AL1 oligomerization domain (**Figure 2A**). Full length GST-AL1 1-352 was co-expressed with truncated AL 1 proteins in baculovirus-infected insect cells and purified on glutathione-sepharose resin. Total extracts and purified proteins were resolved by SDS-PAGE and AL1 was visualized by immunoblotting with AL1 polyclonal antisera. As reported previously, the C-terminal truncation AL1 1-180 (**Fig. 2B**, lanes 3 and 6) co-purified with full length GST-AL1 1-352. Further deletion of the C-terminus to amino acids 168 (lanes 2 and 5) and 158 (lanes 1 and 4) abolished interactions with GST-AL1 protein, demonstrating that the C-terminal limits of the oligomerization domain are between positions 168 and 180. In contrast, N-terminal truncation mutations AL1 134-352 (**Fig. 2C**, lanes 3 and 6), AL1 147-352 (lanes 2 and 5) and AL1 160-352 (lanes 1 and 4) showed a gradual disappearance of interactions with GST-AL1 1-352; the AL1 147-352 and AL1 160-352 interactions varied between weak to background levels. Thus, the N-terminal limit of the oligomerization domain was more difficult to define.

Authentic (i.e., native or natural) AL1 also co-purified with a 60 amino-acid fragment of AL1 fused to GST (GST-AL1 119-180 but not with GST alone (**Fig. 4A**, lanes 1 and 2), demonstrating that sequences between amino acids 119 and 180 are sufficient for AL1 oligomerization. However, additional amino acid contacts can contribute to dimer stability or multimerization. Interactions between GST-AL1 119-180 and the N-terminal truncations of AL1 were then tested. In this assay, deletion to positions 119 (**Fig. 2D**, lanes 1 and 5) and 134 (lanes 2 and 6) did not affect oligomerization, whereas further deletion to positions 147 (lanes 3 and 7) and 160 (lanes 4 and 8) abolished interactions with GST-AL1 119-180.

Together, the results showed that AL1 amino acids 134 to 180 contain the core oligomerization domain and indicated that sequences outside the core contribute additional contacts.

EXAMPLE 3

Alanine substitutions were generated in conserved and charged residues within the core oligomerization domain and adjacent sequences to identify key amino acids that contribute to AL1 interactions (**Figure 3**). Wild type AL1 and proteins with mutations in the core oligomerization domain (E--N140 (Ala4), KEE146 (Ala5), REK154 (Ala6), EKY159 (Ala7), Q-HN165 (Ala8), N-DRI72 (Ala9) and K--E179 (Ala10) were expressed with full length GST-AL1 in insect cells (**Fig. 4A**, top panel) and co-purified on glutathione resin (**Fig. 4A**, bottom panel). Wild-type (lane 1) and mutant AL1 proteins (lanes 3-9) all interacted with GST-AL1. Wild type AL1 did not co-purify with GST alone (lane 2), demonstrating that the interactions were specific for AL1. Similar experiments using GST-AL1 119-180 (**Fig. 4B**) identified one mutant EKY159 (Ala7, lane 6), defective for AL1 interactions. Thus a mutation that impaired AL1 oligomerization was revealed only when co-purified with the core oligomerization domain alone, consistent with the observation that sequences outside the domain contribute to stabilizing interactions.

AL1 EKY159 was assayed for co-purification with truncated GST-AL1 proteins to identify the region that stabilized interactions with the mutant protein. As described above, AL1 EKY159 interacted with full length GST AL1 (**Fig. 4C**, lanes 1 and 5) but not with GST-AL1(119-180) (lanes 4 and 8). AL1 EKY159 interacted with an N-terminal truncation of AL1, GST-AL1(119-352) (lanes 3 and 7) whereas no interaction was detected with a C-terminal truncation of AL1, GST-AL1(1-180) (lanes 2 and 6). These results demonstrate that the C-terminus contributes additional protein contacts outside of the oligomerization domain that can mask the effect of oligomerization mutations within the core domain.

The co-purification assay in insect cells established the limits of the AL1 oligomerization domain and identified amino acids that can be required for protein interactions. The quantitative impact of the mutations on AL1 oligomerization was then analyzed by yeast two-hybrid assays. Expression cassettes for AL1 fused to the GAL4 DNA binding domain and wild type or mutant AL1 fused to the GAL4 activation domain were co-transformed into yeast. Activation of the promoter was assayed by measuring the beta-galactosidase activity in total yeast extracts. Interactions between the mutant and wild type AL1 fusion proteins were then expressed as a percent of wild type AL1/AL1 mediated activation (**Fig. 5A**). Four of the mutations reduced AL1 interactions to 51% (KEE146, Ala5), 57% (REK154, Ala6), 0% (EKY159, Ala7), and 31% (N-DR172, Ala9) of wild type.

In contrast, the mutation Q-HN165 (Ala8) and K-E179 (Ala10) interactions were comparable to wild type. These mutations, located between amino acids 143 and 172, are within the core oligomerization domain. Mutations N-terminal to the core domain, D120 (Ala14), QT130 (Ala2), ND133 (Ala3), and E-N140 (Ala4) were less impaired for AL1 oligomerization, consistent with a role in providing stabilizing contacts to the interaction. The mutation FQ118 (Ala 13) showed no impact on AL1 interactions. Although the mutation RS-R125 (Ala1) is located outside the core domain, AL1 interactions were reduced to 27% of wild type. However, this protein is also impaired for DNA binding. Mutant proteins with reduced AL1 interactions in yeast, REK154 (Ala6), EKY159 (Ala7) and N-DR172 (Ala9) were expressed at levels comparable to wild type, as determined by immunoblot of total protein extracts. Thus, the reduced interactions were not attributable to reduced protein expression levels.

The impact of the AL1 mutations on binding to maize Rb was also analyzed by yeast two-hybrid assays. Expression cassettes for maize Rb (amino acids 214-866) fused to the GAL4 DNA binding domain and mutant AL1 proteins fused to the GAL4 activation domain were co-transformed into yeast. Activation of the promoter was assayed by measuring the beta-galactosidase activity in total yeast extracts. Results were then expressed as a percent of wild type AL1/Rb mediated activation (**Fig. 5B**).

Earlier studies have shown that TGMV AL1 and Rb interact with each other, but the region of the AL1 protein that mediates interaction was not known. The limits of the Rb binding domain were defined by using a baculovirus expression system. Insect cells were co-infected with recombinant baculoviruses corresponding to various AL1 truncations and to a GST fusion with amino acids 214-866 of Maize Rb (GST-mRb). The abilities of the different AL1 truncation to bind GST-mRb were assessed by cofractionation on glutathione-sepharose resin. Total extracts and purified proteins were resolved by SDS-PAGE, and AL1 and GST-mRb were visualized by immunoblotting with AL1 and GST antibodies, respectively. The C-terminal truncation AL1(1-180) copurified with GST-mRb. Further deletion to amino acids 168 and 158 abolished interactions with GST-mRb. Similarly, the N-terminal truncation AL1(101-352) cofractionated with GST-mRb, whereas truncations at positions 110 and 119 were unable to bind GST-mRb. Together, these results mapped the limits of the pRB binding domain between AL1 amino acids 101 and 180. Thus, the C-termini of the pRb binding and oligomerization domains of TGMV AL1 are contiguous, whereas an additional 33 N-terminal amino acids are required for Rb binding (data not shown).

Four of the AL1 mutants were wild type in their ability to bind maize Rb (mRb):

D120 (Ala14), QT130 (Ala2), ND133 (Ala3), E--N140 (Ala4), and K--E179 (Ala10).

Seven mutants displayed reduced Rb binding activity. These mutants fell into two distinct classes. One group, which included the core oligomerization domain mutants REK154 (Ala6), EKY159 (Ala7), N-DR172 (Ala9), was impaired to similar degrees for Rb binding and AL1 oligomerization, suggesting that AL1/AL1 interactions may be a prerequisite for binding to Rb. The second group was more severely impaired for Rb binding than for AL1 oligomerization and thus most likely reflects specific AL1 amino acids that contact Rb. This group included FQ118 (Ala13), RS-R125 (Ala1), AAA136 (Leu), and KEE146 (Ala5). One mutant, Q-HN165 (Ala8), displayed enhanced Rb binding activity.

EXAMPLE 4

The mutations were also assayed for their effect on AL1 functions *in vivo*. Plant expression cassettes for wild type and mutant AL1 were transfected into NT- 1 protoplasts with TGMV B DNA and an expression cassette for AL3. Eleven of the twelve mutants were impaired for the ability to direct viral DNA replication (**Fig. 6A**) when compared to wild type AL1 (lane 1). Only the mutant AL1 K--E179 (Ala10) supported wild type replication levels (lane 13). Mutations within the core oligomerization domain (lanes 9-12) and the conserved sequence between amino acids 117 and 125 (lanes 2-4) abolished replication. The latter group of mutations is within the DNA cleavage and DNA binding domains as well as the enhancer region for AL1 oligomerization and may have pleiotropic effects on AL1 activity. For example, AL1RS-R125 is impaired for DNA binding (results not shown) as well as AL1 oligomerization. In contrast, low levels of DNA synthesis were observed with AL1 mutations in the predicted alpha-helices (lanes 5-8), suggesting that the sequence of this region is less critical for AL1 function.

EXAMPLE 5

Whether replication defective AL1 could interfere with normal viral DNA replication was studied. The present inventors tested N-terminal and C-terminal truncations of wild type AL1, which lack the domains for one or more AL1 activities, and an N-terminal truncation of the AL1 N-DR172 mutant for dominant negative interference of viral replication. The oligomerization domain fused to GST (**Figure 7B**) and a C-terminal truncation of AL1(1-

181) (lane 3) reduced wild type replication, but were significantly less effective than the full length mutant proteins (**Figure 7A**). In addition, replication interference by the N-DR172 mutant was less severe in the N-terminal truncated protein (**Figure 7B**, lane 4) than in the full length AL1

5 A wild type TGMV A replicon (2µg) was transfected into NT- 1 protoplasts in the absence and presence of 20-fold excess (40µg) mutant AL1 expression cassettes. Total DNA was isolated three days post-transfection and analyzed by DNA gel blot hybridization using a radiolabelled TGMV A probe. Mutations in the core oligomerization domain (**Fig. 7A**), REK154 (Ala6, lane 5), EKY159 (Ala7, lane 6), Q-HN165 (Ala8, lane 7), and N-DR172
10 (Ala9, lane 8) reduced single-stranded DNA accumulation 5 to 25% of wild type, and double-stranded DNA accumulation 8 to 29%.

Mutations outside the oligomerization domain, FQ118 (Ala13, lane 2) and D120 (Ala14, lane3), also interfered with replication, reducing single stranded DNA to 3 and 5% of wild type, and double stranded DNA to 1% to 6%, respectively. The mutation RS-R125
15 (Ala1, lane 4) was the least detrimental, reducing single and double stranded DNA by about 50%.

AL1 proteins defective for viral DNA replication interfered with TGMV A DNA replication. These results demonstrate that AL1 mutations that impair replication *in vivo* and affect oligomerization *in vitro* are good candidates for developing transgenic plants resistant
20 to geminivirus infection.

EXAMPLE 6

The ability of the mutants to repress AL1 promoter activity *in vivo* was studied. The AL1 promoter fused to the luciferase reporter gene (*lux*) was transfected into *N. benthamiana*
25 protoplasts either alone or in the presence of plant expression cassettes for wild type and mutant AL1 proteins. In these experiments, wild type AL1 repressed transcription from the AL1 promoter approximately 20-fold. Repression mediated by mutant AL1 proteins was standardized to the percent of wild type repression within each experiment. All of the mutants that reduced viral DNA replication (except for Ala1) also repressed promoter activity 2- to 4-
30 fold higher than wild type AL1. DNA binding is required for repression and Ala1 is a DNA binding mutant. AL1 K--E179 (Ala10) supported normal replication levels and repressed the AL1 promoter similar to wild type AL1. **Figure 6A** (see lane 13) and **Figure 6B**.

EXAMPLE 7

The plasmid pNSB148, which contains the AL1 coding sequence in a pUC118 background, was used as the template for site-directed mutagenesis as described previously (Orozco et al., *J. Virol.* 273:24448-24456 (1998)). The oligonucleotide primers and resulting clones are listed in **Table 5**. Viral DNA fragments containing the mutations were verified by DNA sequence analysis. Viral replicons encoding some of the mutant AL1 proteins were generated, for example, by subcloning Sall/NheI fragments corresponding AL1 amino acids 120-232 from the mutant clones into the same sites into the wild type replicon, pMON1565 (Orozco et al. 1996), to give pNSB954 (K144), pNSB1032 (EE146), pNSB999 (A147Y), pNSB997 (L148), pNSB979 (L148V), pNSB1000 (L148G), and pNSB998 (II151). Alanine substitution mutants are designated [wild type amino acid(s) – final position number]. When the mutation was not to alanine, the mutant amino acid is given after the position number.

Site directed-mutagenesis of CbLCV AL1 was performed using a PCR-based method. Complementary oligonucleotides, Cb-M1 and Cb-M2 (**Table 5**), were used as primers in combination with the M13 universal and reverse primers, respectively, in two separate amplification reactions with pNSB1085 as a template. pNSB1085 contains a CbLCV A DNA fragment (positions 1499 to 33) carrying the AL1 coding sequence in a pUC118 background. The PCR products of Cb-M1/M13-universal and Cb-M2/M13-reverse amplification reactions were isolated from agarose gels and pooled for a new amplification reaction using M13-universal and reverse primers. The PCR product was digested with *Bam*HI and *Bgl*II and cloned into the same sites of a modified pUC118 to give pNSB1097.

Yeast cassettes with the Gal4 DNA binding domain (DBD) were generated using pAS2-1 (Clontech, Palo Alto, CA). Zm214C includes a truncated maize RBR1 coding sequence consisting of the pocket and the C-terminal domains fused the DBD {Ach, 1997 #25}. pNSB736 contains a full-length, wild type TGMV AL1-DBD fusion (Orozco et al., *J. Biol. Chem.* 275:6144-6122 (2000)).

Yeast cassettes containing the Gal4 activation domain (AD) were generated using pACT2 (Clontech). Cassettes for wild type TGMV AL1 (pNSB809) and the mutants, KEE146 (pNSB894) and REK154 (pNSB759), have been described. Cassettes for the TGMV mutants, K144 (pNSB916), E146 (pNSB975), EE146 (pNSB1040), A147Y (pNSB1003), L148 (pNSB1001), L148V (pNSB980), L148G (pNSB1004), and II151 (pNSB1002), were made by replacing the AatII-BamHI fragment of pNSB735 with the equivalents fragments from

pNSB946, pNSB968, pNSB1033, pNSB995, pNSB993, pNSB969, pNSB996 and pNSB994, respectively.

The C1 open reading frame was amplified from a TYLCV (Dominican Republic) genomic clone (Monsanto) using the oligonucleotides, 5'-

5 GGACACCGATTggaTcCAgCATGCCTC (**SEQ ID NO: 103**) and
CCACAGTCgAatTCCCCggGCTTACGC (**SEQ ID NO: 104**). (The lowercase letters indicate mutated nucleotides.) The resulting PCR product (positions 1686 to 12 of the TYLCV-DR genome) was digested with *Bam*HI and *Sma*I and cloned into pUC119 to give pTYLC78. The corresponding yeast AD vector was constructed by cloning the *Bam*HI
10 (trimmed)/*Sma*I C1 fragment from pTYLC78 into pACT2 digested with *Sma*I to give pTYLC102.

pCPCbLCVA.001, which has a single copy of CbLCV A DNA (Turnage et al., Plant J. 30:107-114 (2002)), was modified by PCR mutagenesis using the oligonucleotide 5'-
CCTAAATAagatcTACAAGgATcCCACGAAACCCTA (**SEQ ID NO: 105**) to introduce a
15 *Bam*HI site at the 5' end of the AL1 open reading frame. The resulting clone, pNSB900, was digested with *Bam*HI, and the fragment containing the full-length AL1 sequence was cloned into the *Bam*HI site of pACTII to give pNSB958. The *Nco*I fragment from pNSB958 encoding amino acids 2-178 of AL1 was then subcloned into pACTII to give pNSB974. The L145A mutation was introduced into the CbLCV AL1 coding sequence of pNSB900 by using
20 the oligonucleotide 5'-GTGTGGAAGAGGCGGgccGCAATTATAAGGGC (**SEQ ID NO: 106**) for PCR mutagenesis. A mutant fragment from the resulting plasmid (pNSB1097) with *Aat*II/*Nco*I (repaired) ends was subcloned into pNSB974 with *Aat*II/*Xho*I (repaired) ends to give pNSB1114.

Interactions between the Gal4 fusion proteins were evaluated in *Saccharomyces cerevisiae* strain Y187 by measuring β -galactosidase activity. Protein concentrations were measured by Bradford assays (Biorad, Hercules, CA). The enzyme specific activity (1 unit = 1.0 μ m product/min at pH7.3 at 37°C) was determined using purified β -galactosidase (Sigma, St. Louis, MO) as the standard. The different constructs were tested in a minimum of four independent transformants in at least two experiments. The relative activities of the mutant
30 proteins were normalized against wild type AL1, which was set to 100%.

TGMV A replicons carrying mutant AL1 coding sequences were made using pMON1565, a pUC-based plasmid that contains 1.5 copies of wild type TGMV A DNA (Orozco et al., *J. Virol.* 270:148-158 (1996)). The mutant replicons in Table 5 were generated

by replacing the *SalI/NheI* fragment encoding AL1 amino acids 120 to 313 of pMON1565 with the equivalent fragment of mutant AL1 open reading frames described above. The plasmid, pTG1.4B, which includes 1.4 copies of wild type TGMV B has been described (Fontes et al., *Plant Cell* 6:405-416 (1994)).

5 For replication assays, protoplast were isolated from *Nicotiana tabacum* (BY2) suspension cells, electroporated and cultured according to published methods (Fontes et al., *J. Biol. Chem.* 269:8459-8465 (1994)) Cells were transfected with 5 µg of wild type or mutant TGMV A replicon DNA and 25 µg of sheared salmon sperm DNA. Total DNA was extracted 72h after transfection, digested with *DpnI* and *XhoI*, and examined for double- and single-
10 stranded viral DNA accumulation by DNA gel blot analysis using a TGMV A specific probe (Fontes et al., *Plant Cell* 6:405-416 (1994)). Viral DNA was quantified by phosphorimage analysis. Each protoplast assay was performed in at least three independent experiments.

For infectivity assays, *N. benthamiana* plants at the 6-leaves stage were inoculated using a biolistics device (Nagar et al., *Plant Cell* 7:705-719 (1995)). Wild type or mutant TGMV A
15 replicon DNA (10 µg) was precipitated onto 1 µm microprojectiles in the presence of a wild-type TGMV B replicon (pTG1.4B) and bombarded into plants. Total DNA was extracted from leaf tissue 14 days after bombardment (Dellaporta et al., 1983), digested with *XhoI*, and analyzed on DNA gel blots.

The RBR1 binding domain of AL1 has been mapped to between amino acids 101-180 and
20 mutations in residues KEE146 have been shown to impair binding. The KEE146 mutant contains three consecutive alanine substitutions, which may act alone or together to confer the mutant phenotype. To better understand the basis of the phenotype, four additional alanine substitution mutants, K144, E145, E146, and EE146 (**Figure 8A**), were produced. The mutant AL1 open reading frames were fused to the coding sequence of Gal4 activation
25 domain (AD) and expressed in yeast. Mutant proteins accumulated to levels comparable to an AD-wild type AL1 fusion, as determined by immunoblot analysis. The AL1 oligomerization properties of the mutant proteins were assessed in two hybrid assays using a wild type TGMV AL1 protein fused to the Gal4 DNA binding domain (DBD). The influence of the mutations on AL1/AL1 interactions was minimal (**Figure 8B**, right) and not statistically
30 significant, indicating that none of the mutations have a global effect on AL1 function.

The impact of the mutations on AL1-RBR1 interactions was analyzed in two hybrid assays using a truncated version (Zm214C) of maize RBR1 from amino acid 214 to the C-terminus fused to the Gal4 DBD (Ach et al., *Mol. Cell Biol.* 17:5077-5086 (1997)). This

region contains the A/B pocket domain and the C-terminal domain of RBR1. The E145, E146 and EE146 mutations did not alter AL1/RBR1 binding significantly (**Figure 8B**), suggesting that the E residues are not essential for wild type binding activity. In contrast, the K144 mutation reduced RBR1 interactions to 42% of the wild type levels (**Figure 8B**), indicating that this residue is required for full binding activity. However, the reduction in RBR1 binding activity was less for K144 than KEE146 (16%; **Figure 8B**), thereby uncovering a role for one or both of the E residues in interaction with RBR1.

Mutations in the helix 4 motif of AL1 impair RBR interactions

The KEE sequence constitutes the first three residues of an 11 amino acid motif designated as Helix 4 (**Figure 8A**). The Helix 4 motif is conserved across all geminivirus replication proteins both with respect to amino acid sequence and its predicted α -helical structure. Previous studies have shown that the RBR1 binding activity of an REK154 mutant, which contains three alanine substitution in the last three amino acids of the motif, is reduced 2-fold (**Figure 8B**). To further explore the role of the Helix 4 motif in RBR1 binding, alanine substitutions were generated at the conserved L148 and II151 residues and a tyrosine substitution was generated at the invariant A147 position. The Q149 position, which is highly variable, was not mutated.

The mutant AL1 coding sequences were fused to the coding sequence of Gal4 AD and analyzed in yeast two hybrid assays as described herein. The A147Y and II151 mutants displayed significantly lower RBR1 binding activities than wild type AL1 (**Figure 8B**). However, these mutations also reduced AL1 oligomerization activity (**Figure 8B**, right), indicating that their effects are not specific for RBR1 binding. In contrast, the L148 mutation reduced the AL1/RBR1 interactions to 25% of wild type levels without a concomitant loss in AL1 oligomerization activity (**Figure 8B**), thereby establishing the specificity of the mutation for RBR1 binding. Together, these results indicate that several of the amino acid residues in the Helix 4 motif are important for both AL1/RBR and AL1/AL1 interactions in yeasts, and that both the KEE146 and the L148 residues contribute specifically to RBR1 binding.

The KEE146 mutation alters the level of TGMV DNA accumulation in cultured cells and the tissue specificity and symptoms of TGMV infection in plants. To determine if mutations in other Helix 4 residues also impact these viral processes, additional mutants were analyzed in transient replication and infectivity assays. The mutations were transferred into the AL1 open reading frame of a TGMV A replicon and viral DNA accumulation in *Nicotiana tabacum* BY-2 protoplasts was assessed on DNA gel blots. The K144 (**Figure 9A**, lane 2)

and EE146 (lane 3) mutants replicated to wild type levels (lane 1). A similar result was observed with mutants E145 and E146 (data not shown). The L148 mutant (**Figure 9A**, lane 5) also supported viral DNA synthesis but at levels significantly lower than wild type TGMV A (lane 1). This reduction in DNA accumulation (13% of wild type levels) was observed for both double- and single-stranded forms of TGMV DNA. In contrast, the A147Y (**Figure 9A**, lane 4) and II151 (lane 5) mutants, both of which severely impaired AL1 oligomerization (**Figure 8B**), failed to replicate to detectable levels in cultured cells. Similar viral DNA accumulation patterns were observed when BY-2 protoplasts were co-transfected with a TGMV B replicon and with plant expression cassettes for AL1 and AL3.

Plant infection experiments were carried out by co-bombarding either wild type or mutant A component DNA with a TGMV B replicon onto *N. benthamiana* plants. Plants inoculated with the wild type virus developed clear symptoms by 6-7 days post-inoculation, exhibiting leaf curling, general chlorosis and stunting of new growth (**Figure 9B**, wt). The K144, E145, E146 and EE146 mutants developed wild type symptoms with a similar timing (data not shown), indicating that these mutations do not visibly alter the infection process. In contrast, the L148 mutant produced milder symptoms that appeared 14-21 days post infection. L148-infected plants displayed no stunted growth or leaf curling, and only developed chlorosis along the veins (**Figure 9B**, L148). These symptoms, which resemble those of KEE146-infected plants, were observed in all 12 inoculated plants and were maintained over a 5-week infection period. Mutants A147Y and II151 produced no detectable symptoms even at 5 weeks post-inoculation, consistent with their inability to replicate in tobacco protoplasts.

TGMV DNA accumulation was also examined in *N. benthamiana* plants inoculated with either wild type or mutant virus. Total DNA was isolated from systemically infected leaves 14 days post-inoculation and analyzed on DNA gel blots using a TGMV A probe. Viral DNA was detected in extracts of plants infected with all mutant viruses that produced symptoms (**Figure 9C**), but not in asymptomatic plants inoculated with the A147Y (lanes 8-10) and II151 (lanes 14-16) mutants. Plants infected with K144 (**Figure 9C**, lanes 2-4) or EE146 (lanes 5-7) contained wild type levels of single- and double-stranded viral DNA (lane 1). The same results were obtained with E145- and E146-infected plants. In contrast, both DNA forms were reduced in L148-inoculated plants relative to wild type (**Figure 9C**, cf. lane 1 and 11-13). DNA gel blot analysis at 7, 14, and 21 days post inoculation showed that the differences in TGMV DNA levels between L148- and wild-type TGMV-infected plants are stable over time. A similar reduction in the accumulation of viral DNA was previously

observed in plants infected with the KEE146 mutant. However, these earlier experiments established that there is not a direct relationship between attenuation of symptoms and reduction of virus DNA accumulation during infection. Instead, the attenuated symptoms caused by the L148 mutation are likely to be due to reduced RBR1 binding as has been
5 hypothesized for KEE146.

Even though the phenotypes caused by the KEE146 and L148 mutations are very similar, the L148 mutation is the only single-site substitution in the RBR1 binding domain of AL1 that produced a clear effect. The role of L148 in AL1/RBR1 interactions was thus examined in greater detail. The L148 residue may facilitate RBR1 binding by contributing molecular
10 contacts and/or by stabilizing the predicted structure of the Helix 4 motif. To address these possibilities, a series of amino acids were substituted at position L148 with different side chains and tendencies to occur in α -helices. The impact of the different mutations on the RBR1 binding and oligomerization activities of TGMV AL1 was analyzed in yeast dihybrid assays (**Figure 10**). A L148M mutation had no detectable effect on RBR1 binding activity,
15 whereas a L148I substitution resulted in a moderate reduction. Like the L148 mutant, the L148V and L148G mutants displayed significantly less RBR1 binding activity than wild type AL1. In general, the binding activities of the mutants declined with the probability of the substituted amino acid to occur in an α -helix (O'Neil et al., *Science* 250:646-51 (1990)). However, the low activity of the L148 mutant, which is predicted to readily form an α -helical
20 structure, supports the involvement of side-chain contacts. Together, these results suggested that L148 contributes both structural and specific contacts to RBR1 binding. None of the mutations had a strong effect on AL1 oligomerization activity, indicating that the reduced RBR1 binding activities are not due to a general destabilization of AL1.

To determine if RBR1 binding is a general property of begomovirus replication proteins, the ability of C1/AL1 proteins of other begomoviruses to interact with RBR1 was examined.
25 The studies employed two geminiviruses, tomato yellow leaf curl virus (Dominican Republic isolate; TYLCV-DR) and cabbage leaf curl virus (CbLCV), which are evolutionarily distant from TGMV and from each other. TYLCV-DR, which has a single genome component, is representative of Old World begomoviruses (Salati et al., *Phytopathology* 87:S84 (1997)).
30 CbLCV is from a small group of New World begomoviruses whose AL1 proteins lack a highly conserved sequence of unknown function between the DNA cleavage motif III and the predicted Helix 3 (Hill et al., *Virology* 250:283-292 (1998)). Gal4 AD fusions corresponding to full-length TYLCV C1 and CbLCV AL1 were generated and tested for interaction with a

DBD-RBR1 fusion in yeast. The RBR1 binding activity of TYLCV C1 was similar to that detected for TGMV AL1 in parallel assays (**Figure 11A**). In contrast, no colonies carrying the expression cassette corresponding to the CbLCV full-length AL1 fusion were recovered. Similar problems have been encountered in bacterial and insect cell systems with full-length CbLCV AL1, indicating that its expression is detrimental. To overcome this problem, a Gal4 AD fusion, corresponding to amino acids 2-178 of CbLCV AL1, was generated. Using this fusion, a reduced but significant level of RBR1 binding by the CbLCV AL1 N-terminus was detected (**Figure 11A**).

The AL1/C1 sequences from 78 begomoviruses of both Old World and New World descent were compared to derive a consensus sequence for the Helix 4 motif (**Figure 11B**). These comparisons revealed that the motif consists of a conserved hydrophobic core flanked by charged residues. The core includes an invariant alanine residue followed by a leucine in 67 of the examined AL1/C1 proteins and methionine in the remaining 11 proteins. The L/M position corresponds to L148 in TGMV and is represented by a leucine in both TYLCV and CbLCV. The substitution of an alanine at position L145 in CbLCV AL1 was examined for the ability to impair RBR1 binding analogous to the TGMV L148 mutant. The L145A mutation was introduced into Gal4 AD fusion vector carrying amino acids 2-178 of the CbLCV AL1 coding sequence and tested for RBR1 binding in yeast dihybrid assays. As shown in **Figure 11A**, the CbL145A mutation caused a significant reduction in RBR binding activity. The CbLCV L145A and TGMV L148 mutations reduced RBR binding to 23% (**Figure 11A**) and 25% (**Figure 8B**) of their respective wild type controls. Together, these data show that diverse begomovirus replication proteins interact with RBR through a conserved motif.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

TABLE 1
TGMV Rep Retinoblastoma Binding Mutants and Phenotypes

Rep Mutant (a)	Rep1 (b)	oligomer. (c)	Rep/Rb (d)	Repress (e)	Dom.Neg (f)
Ala1 (SEQ ID NO: 56)	neg.	27	20	30	+
Ala4+5 (SEQ ID NO: 78)	neg.	88	45	322	+
Ala6 (SEQ ID NO: 66)	neg.	57	69	223	+
Ala7 (SEQ ID NO: 68)	neg.	0	31	234	+
Ala13 (SEQ ID NO: 52)	neg.	95	23	259	+
Leu (SEQ ID NO: 76)	neg.	30	8	206	+

TABLE 2
TGMV Rep Oligomerization Mutants and Phenotypes

Rep Mutant (a)	Rep1 (b)	oligomer. (c)	Repress (e)	Dom.Neg. (f)
Ala13 (SEQ ID NO: 52)	neg.	95	259	+
Ala14 (SEQ ID NO: 54)	neg.	67	328	+
Ala1 (SEQ ID NO: 56)	neg.	27	38	+
Ala4+5 (SEQ ID NO: 78)	neg.	88	322	+
Ala6 (SEQ ID NO: 66)	neg.	57	223	+
Ala7 (SEQ ID NO: 68)	neg.	0	234	+
Ala8 (SEQ ID NO: 70)	neg.	102	212	+
Ala9 (SEQ ID NO: 72)	neg.	31	242	+

(a) Rep sequence mutations located in the TGMV AL1 region from amino acids 120-179 (see **Figure 1**)

(b) Rep1 = the capacity for each mutant to support TGMV replication in tobacco protoplasts.

(c) oligomer. = the oligomerization activity of each mutant relative to wild type (100) in yeast dihybrid assays.

(d) Rep/Rb = the Rb binding activity of each mutant relative to wild type (100) in yeast dihybrid assays (maize Rb).

(e) Repress = the ability of each mutant to repress the AL1 promoter relative to wild type Rep (100) in tobacco protoplasts.

(f) Dom.Neg. = the capacity of each mutant to interfere with replication of wild type TGMV A component in tobacco protoplasts.

TABLE 3

Name	Amino Acid substitution sites	SEQ. ID NO.
Alanine Substitutions		
Ala1	RS-R125	56
Ala2	QT130	58
Ala3	ND133	60
Ala4	E--N140	62
Ala4+5	E--N140 + KEE146	78
Ala5	KEE146	64
Ala6	REK154	66
Ala7	EKY159	68
Ala8	Q-HN165	70
Ala9	N-DR172	72
Ala10	K--E179	74
Ala13	FQ118	52
Ala14	D120	54
Leucine Substitutions		
Leu	AAA136	76

TABLE 4

AL1 Mutations

Mutation	Oligonucleotide	SEQ ID NO:	Baculovirus Vector	Yeast GAL4-AD	Plant Expression
Wt	N/A				
FQ118 (Ala13)	CACTTCGACCGTCGACCGCGGCTTCTCCCCA	26	pMON1680	pNSB809	pMON1549
D120 (Ala14)	CACTTCGGCCGGCGACCGCGGCTTCTCCCCA	27	N/A	pNSB872	pNSB866
RS-R125 (Ala1)	GCAACCTCCTGcAGCggcgccACCGTCGACCTGGA	28	N/A	pNSB871	pNSB865
QT130 (Ala2)	CAGCGTCGTTgcaGcTgcGCAACCTCCTCTAGCA	29	N/A	pNSB786	pNSB695
ND133 (Ala3)	CTGCTGCAGCGGcGcAGATGTTTGGCAA	30	pNSB603	pNSB788	pNSB696
E--N140 (Ala4)	GGAAGAAAGCAgcTAAACGcGcCgCTGCAGCGTCGT	31	pNSB604	pNSB970	pNSB670
KEE146 (Ala5)	TCTGCAGGGCTgCggCgcGGAAAGCAATTAA	32	pNSB605	ND	pNSB640
REK154 (Ala6)	TTCCTGGGATTgcccGcgcAAATTATCTGCAGGG	33	pNSB605	ND	pNSB641
EKY159 (Ala7)	GAACTGAAATAAAggggccgCTGGGATTTCTCTC	34	pNSB605	pNSB759	pNSB671
Q-HN165 (Ala8)	GCTATTTAGAGcGgcGAACgcAAATAAATAATTTTCTGGGAT	35	pNSB607	pNSB760	pNSB672
N-DR172 (Ala9)	ATCAAAATATCgcAgCTAgcgcGCTATTAGATTGTG	36	pNSB608	pNSB761	pNSB698
K--E179 (Ala10)	GAAGCCATGGcGcGGAGTCgcATCAAAATATCC	37	pNSB609	pNSB762	pNSB707
AAA136 (Leu)	GAAGCAAATTTAAgGCCTCTTagTagAgGTCGTTAGATG	38	pNSB610	pNSB763	pNSB697
E--N140 + KEE146 (Ala4+5)	TCTGCAGGGCTgCggCgcGGAAAGCAgcTAAACGCgCgCTGCAGCGTCGT	39	pNSB743	pNSB785	pNSB676
			pNSB659	pNSB757	pNSB648
					Viral Replicon
K144	TCTGCAGGGCTTCTTCgcGGAAGAAGCATTAA	40		pNSB916	pNSB954
E145	TCTGCAGGGCcTCTgCTTTGGAAGAAGCATTAA	41		pNSB917	
E146	CTGCAGGGCTgCTTCTTTTGGAAAGCA	42		pNSB975	
EE146	TCTGCAGGGCcgcCgCTTTGGAAAGAAGCATTAA	43		pNSB1040	pNSB1032
A147Y	TAAATTATCTGaAGGaaTTCTTCTTTTGGAAAGAAGCATTAA	44		pNSB1003	pNSB999
L148	TAAATTATCTGCgaGCTTCTTCTTTTGGAAAGAAGCATTAA	45		pNSB1001	pNSB997

L148V L148V*	ATTATCTGCACGGCCTCTTCTTTTGGGAAGAAGCATTAA TAATTATCTGaAcGGCTTCTTCTTTGGGAAGAAGCATTAAAC	46			pNSB980 pNSB1039	pNSB979 pNSB1030
L148G	CTAATTATCTGgcGGCTTCTTCTTTTGGGAAGAAGCATTAA	47			pNSB1004	pNSB1000
III151	TTTCTCTCTAgcTgcCTGaAGGGCTTCTTCTTTGGAAGA	48			pNSB1002	pNSB998

Table 5

Constructs

Mutation	Mutagenesis oligonucleotides	SEQ ID NO:	Yeast dihybrid vectors GAL4-AD-AL1 GAL4-BD	Viral Replicons
TGMV AL1			pNSB809	pMON1565
KEE146			pNSB894	
K144	TCTGCAGGGCTTCTTCgcGGAAGAAGCATTAA	40	pNSB916	pNSB954
E145	TCTGCAGGGCcTCTgCTTTGGAAGAAGCATTAA	41	pNSB917	
E146	CTGCAGGGCTgCTTCTTTGGAAGAAGCA	42	pNSB975	
EE146	TCTGCAGGGCcggCcgCTTTGGAAGAAGCATTAAAC	43	pNSB1040	pNSB1032
A147Y	TAATTATCTGaAGGtaTTCTTCTTTGGAAGAAGCATTAA	44	pNSB1003	pNSB999
L148	TAATTATCTGcgaGCTTCTTCTTTGGAAGAAGCATTAA	45	pNSB1001	pNSB997
L148V	ATTATCTGCACGGCcTCTTCTTTGGAAGAAGCATTAA	46	pNSB980	pNSB979
L148V*	TAATTATCTGaAcGGCTTCTTCTTTGGAAGAAGCATTAAAC	47	pNSB1039	pNSB1030
L148M	pNSB979 revertant		pNSB1112	
L148G	CTAATTATCTGgcGGCTTCTTCTTTGGAAGAAGCATTAA	48	pNSB1004	pNSB1000
L148I	pNSB1030 revertant		pNSB1079	
II-150-AA	TTTCTCTCTAgcTgcCTGaAGGGCTTCTTCTTTGGAAGA	49	pNSB1002	pNSB998
REK154			pNSB759	
CbLCV AL1			pNSB901	pNSB909
Cb1-207			pNSB974	
Cb1-207, L145A	GTGTGGAAGAGGGGgcGCAATTATAAGGGC	106	pNSB1114	
TYLCV-DR C1			pTYLC102	pTYLC103

Maize RBR1						214C	